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CONTENTS OF VOLUME 169

No. 1, JUNE, 1947

	PAGE
KOCHAKIAN, CHARLES D., and VAIL, VIRGINIA N. The effect of adrenalectomy, adrenal cortical hormones, and testosterone propionate plus adrenal cortical extract on the arginase activity of the liver and kidney of the rat	1
FISHMAN, WILLIAM H. β -Glucuronidase: its relation to the action of the estrogenic hormones	7
POTTER, VAN R. The assay of animal tissues for respiratory enzymes. VI. Further studies on oxidative phosphorylation	17
SIMONSEN, DAISY G., WESTOVER, LEOLA M., and WERTMAN, MAXINE. The determination of serum magnesium by the molybdivanadate method for phosphate	39
TAUROG, ALVIN, and CHAIKOFF, I. L. The metabolic interrelations of thyroxine and diiodotyrosine in the thyroid gland as shown by a study of their specific activity-time relations in rats injected with radioactive iodine	49
ROGERS, LORENE LANE, and SHIVE, WILLIAM. Biochemical transformations as determined by competitive analogue-metabolite growth inhibitions. VI. Prevention of biotin synthesis by 2 oxo 4-imidazolidinecaproic acid	57
DITTMER, KARL, and DU VIGNEAUD, VINCENT. Antibiotin activity of imidazolidone aliphatic acids	63
HORN, MILLARD J., JONES, D. BREWSE, and BLUM, AMOS E. Microbiological determination of lysine in proteins and foods	71
CARTER, HERBERT E., HAINEs, WILLIAM J., LEDYARD, W. E., and NORRIS, WILLIAM P. Biochemistry of the sphingolipides. I. Preparation of sphingolipides from beef brain and spinal cord	77
JOHNSON, R. M., and BAUMANN, C. A. Studies on the reaction of certain carotenoids with antimony trichloride	83
SIMMONDS, SOFIA, TATUM, E. L., and FRUTON, JOSEPH S. The utilization of phenylalanine and tyrosine derivatives by mutant strains of <i>Escherichia coli</i>	91
TAUSSKY, HERTHA H., and SHORR, EPHRAIM. A microcolorimetric method for the determination of citric acid	103
COHEN, PHILIP P., and MCGILVERy, R. W. Peptide bond synthesis II The formation of <i>p</i> -aminohippuric acid by liver homogenates	119
HACK, M. H. Estimation of the phospholipides in human blood	137
GI TMANN, HELMUT R., JANDORF, BERNARD J., and BODANSKY, OSCAR. The rôle of pyridine nucleotides in the reduction of methemoglobin	145
BOXER, GEORGE E., JELINEK, VIOLA C., and LEGHORN, PATRICIA M. The colorimetric determination of streptomycin in clinical preparations, urine, and broth	153
HUFFMAN, MAX N. 16-Substituted steroids. III. The partial synthesis of estriol	167
SCHOLANDER, P. F., FLEMISTER, SARAH C., and IRVING, LAURENCE. Microgasometric estimation of the blood gases. V. Combined carbon dioxide and oxygen	173
FRIEDKIN, MORRIS, and LEHNINGER, ALBERT I. The synthesis of phosphomalic acid	183

HOFMANN, KLAUS, WINNICK, THEODORE, and AXELROD, A. E. The use of Radey's nickel in a differential assay for oxybiotin and biotin	191
AXELROD, A. E., FLINN, BARBARA C., and HOFMANN, KLAUS. The metabolism of oxybiotin in yeast	195
PORTIS, RICHARD A., and ALTMAN, KURT I. The action of proteolytic enzymes and protaminase on salmine sulfate	203
HUEBNER, CHARLES F., and JACOBS, WALTER A. The aconite alkaloids. XVI. On staphisine and the hydrocarbon obtained from its dehydrogenation	211

Letters to the Editors

DOBRINER, KONRAD, LIEBERMAN, SEYMOUR, HARITON, I., SARETT, LEWIS HASTINGS, and RHOADS, C. P. The isolation of Δ^9 -etiocholesterol-3(α)-one-17 from human urine	221
MAISON, HAROLD L., POWER, MARSCHELLE H., RYNEARSON, E. H., CIARANELLI, L. C., LI, CHOH HAO, and EVANS, HERBERT M. Results of administration of anterior pituitary adrenocorticotrophic hormone to a normal human being	223
GONÇALVES, JOSE M., PRICE, VINCENT E., and GREENSTEIN, JESSE P. Enzymatic hydrolysis of acetyldehydroalanine	225
GEYER, R. P., NATH, II, BARKI, V. H., ELVEHJEM, C. A., and HART, E. B. The vaccenic acid content of various fats and oils	227
HUTCHINGS, B. L., and BOGGIANO, E. Oleic acid as a growth factor for various lactobacilli	229
WARE, ARNOLD G., GUEST, M. MASON, and SEEGER, WALTER H. A factor in plasma which accelerates the activation of prothrombin	231
GOLLUB, MIRIAM C., and VENNESLAND, BIRGIT. Fixation of carbon dioxide by a plant oxalacetate carboxylase	233
GRAY, CHARLES H., and HOLT, LEWIS B. The porphyrin produced by diphtheria bacillus	235

No 2, JULY, 1947

VICKERY, HUBERT BRADFORD. Rules for the nomenclature of the natural amino acids and related substances	237
BORSOOK, HENRY, and DUBNOFF, JACOB W. Methionine formation by trans-methylation <i>in vitro</i>	247
CSONKA, FRANK A., DENTON, CHARLES A., and RINGEL, S. J. The methionine and cystine content of hen's eggs	259
FRUTON, JOSEPH S., SIMMONDS, SOFIA, and SMITH, VIRGINIA A. The action of <i>Escherichia coli</i> on acetyldehydroamino acids	267
MICHEL, HARRY O., and SCHEINBERG, I. HERBERT. The effects of cytochrome c on anoxic and cyanide poisoned rats	277
JAFFÉ, WERNER G., and ELVEHJEM, C. A. Fractionation of growth-stimulating factor in liver	287
BUHLER, HENRY J., SCHANTZ, E. J., and LAMANNA, CARL. The elemental and amino acid composition of crystalline <i>Clostridium botulinum</i> type A toxin	295
SIZER, IRWIN W. The action of tyrosinase on certain proteins and products of their autolysis	303
LENS, J. The inactivation of insulin solutions	313
BOLOMEY, RENÉ A. Oxidative decomposition of vitamin A. I. Stability of vitamin A towards oxidation and irradiation	323
BOLOMEY, RENÉ A. Oxidative decomposition of vitamin A. II. Absorption spectrophotometry of oxidized vitamin A	331

MORGAN, J. F., and CAMPBELL, M. E. A rapid method for the production and isolation of penicillinase	337
BEILER, J. M., and MARTIN, GUSTAV J. Inhibition of the action of tyrosine decarboxylase by phosphorylated desoxyypyridoxine	345
ZAMECNIK, PAUL C., and STEPHENSON, MARY L. A manometric method for determining the kinetics of an enzymatic hydrolysis of peptides	349
FRANTZ, IVAN D., JR., and STEPHENSON, MARY L. A study of the reaction kinetics of a catheptic enzyme with a decarboxylase as indicator	359
STUMPF, P. K. A colorimetric method for the determination of desoxyribonucleic acid	367
SCHOFIELD, FRANCIS A., and LEWIS, HOWARD B. A comparative study of the metabolism of α -alanine, β -alanine, serine, and isoserine II Glycogen content of the liver after oral administration of the amino acids	373
PETERMANN, MARY L., YOUNG, NELSON F., and HOGNESS, KATHARINE R. A comparison of the Howe and the electrophoretic methods for the determination of plasma albumin	379
POLIS, B. DAVID, and MEYERHOF, OTTO. Studies on adenosinetriphosphatase in muscle. I Concentration of the enzyme on myosin	389
BUCHANAN, JOHN M., SAKAMI, WARWICK, GURIN, SAMUEL, and WILSON, D. WRIGHT. Intermediates in the biological oxidation of isotopic acetoacetate	403
BUCHANAN, JOHN M., SAKAMI, WARWICK, and GURIN, SAMUEL. A study of the mechanism of fatty acid oxidation with isotopic acetoacetate	411
MILLVILLE, DONALD B., RACHELLE, JULIAN R., and KELLER, ELIZABETH B. A synthesis of methionine containing radiocarbon in the methyl group	419
FRANKLIN, A. L., STOKSTAD, E. L. R., BELT, MARGARET, and JUKES, THOMAS H. Biochemical experiments with a synthetic preparation having an action antagonistic to that of pteroylglutamic acid	427
DEUTSCH, H. F. A study of whey proteins from the milk of various animals	437

Letters to the Editors

FISHMAN, WILLIAM H., and ANLYAN, A. J. The presence of high β -glucuronidase activity in cancer tissue	449
LARDY, HENRY A., POTTER, RICHARD L., and ELVEHJEM, C. A. The rôle of biotin in bicarbonate utilization by bacteria	451
SHIVE, WILLIAM, and ROGERS, LORENE LANE. Involvement of biotin in the biosynthesis of oxalacetic and α -ketoglutaric acids	453
SHORB, MARY S. Unidentified growth factors for <i>Lactobacillus lactis</i> in refined liver extracts	455
RHYMER, IONE, WALLACE, G. I., BYERS, L. W., and CARTER, HERBERT E. The antistreptomycin activity of lipositol	457
ARONOFF, S., BARKER, H. A., and CALVIN, M. Distribution of labeled carbon in sugar from barley	459
BORSOOK, HENRY, and DUBNOFF, JACOB W. Ornithine-catalyzed urea formation in liver homogenate	461
ALTMAN, KURT I., and EVANS, E. A., JR. Enzymic synthesis of triphosphopyridine nucleotide in cell-free extracts	463
GALSTON, ARTHUR W. Synergism between indoleacetic and nicotinic acids in a plant growth inhibition	465

No. 3, AUGUST, 1947

BLOCH, KONRAD, and RITTENBERG, D. The metabolism of acetyl amino acids...	467
LAVINE, THEODORE F. The formation, resolution, and optical properties of the diastereoisomeric sulfoxides derived from L-methionine ..	477
BIRD, ROBERT M. Glycolysis in human blood	493
AMES, STANLEY R. Effect of calcium on the inhibition of the succinic oxidase system by <i>d</i> - α -tocopheryl phosphate	503
QUAIFE, MARY LOUISE. Tocopherols (vitamin E) in milk: their chemical determination and occurrence in human milk	513
FRANKLIN, A. L., LI, CHOH HAO, and DUNN, MAX S. The amino acid composition of anterior hypophyseal growth hormone	515
YUDKIN, WARREN II., and FRUTON, JOSEPH S. On the proteolytic enzymes of animal tissues. VI. Dehydropeptidase	521
GUSTAVSON, K. H. Note on the reaction of formaldehyde with collagen	531
FOICH, JORDI, and LAUREN, MIRIAM. Estimation of potassium in biological materials as potassium phosphotungstate	539
SCHOLANDER, P. F., and EVANS, II. J. Microanalysis of fractions of a cubic millimeter of gas	551
SCHOLANDER, P. F., and IRVING, LAURENCE. Micro blood gas analysis in fractions of a cubic millimeter of blood ..	561
CHINARD, FRANCIS P., and VAN SLYKE, DONALD D. Comparison of a modified Folin photometric procedure and the ninhydrin manometric method for the determination of amino acid nitrogen in plasma	571
MAJOOR, C. L. H. The possibility of detecting individual proteins in blood serum by differentiation of solubility curves in concentrated sodium sulfate solutions. II. Comparison of solubility curves with results of electrophoresis experiments	583
MILNE, JOHN. Serum protein fractionation: a comparison of sodium sulfate precipitation and electrophoresis	595
LORING, HUBERT S., CARPENTER, F. H., and ROLL, PAUL M. The hydrolysis of yeast ribonucleic acid by ribonucleinase. I. The extent of hydrolysis and the preparation of ribonucleinase-resistant fractions after ribonucleinase treatment	601
DEMEIO, R. H., and HENRIQUES, F. C., JR. Tellurium. IV. Excretion and distribution in tissues studied with a radioactive isotope ..	609
LI, CHOH HAO, KALMAN, C., and EVANS, HERBERT M. The effect of the hypophyseal growth hormone on the alkaline phosphatase of rat plasma	625
RABINOWITZ, JESSE C., and SNELL, ESMOND E. The vitamin B ₆ group. XI. An improved method for assay of vitamin B ₆ with <i>Streptococcus faecalis</i>	631
RABINOWITZ, JESSE C., and SNELL, ESMOND E. The vitamin B ₆ group. XII. Microbiological activity and natural occurrence of pyridoxamine phosphate	643
JERVIS, GEORGE A. Studies on phenylpyruvic oligophrenia. The position of the metabolic error	651
GJESSING, ERLAND C., and CHANUTIN, ALFRED. An electrophoretic study of plasma and plasma fractions of normal and injured rats	657
MORRISON, PETER R. An automatic apparatus for the determination of oxygen consumption	667
OLSON, KENNETH J., ELY, RAY E., and REINEKE, E. P. An improved method for the determination of thiouracil.	681

DANIEL, LOUISE J., NORRIS, L. C., SCOTT, M. I., and HEUSER, G. F. Growth inhibition of bacteria by synthetic pterins. I. Studies with <i>Streptococcus faecalis</i> , <i>Lactobacillus casei</i> , and <i>Lactobacillus arabinosus</i>	689
HANAHAN, DONALD J., and CHAIKOFF, I. L. A new phospholipide-splitting enzyme specific for the ester linkage between the nitrogenous base and the phosphoric acid grouping	699
HODGE, HAROLD CARPENTER, MACLACHLAN, P. L., BLOOR, W. R., WELCH, EILEEN A., KORNBERG, SYLVIA LEVY, and FALKENHEIM, MARLENE. Lipides of the fasting mouse. III. Phospholipide to neutral fat relationship in the blood lipides	707
FALKENHEIM, MARLENE, NEUMAN, WILLIAM F., and HODGE, HAROLD CARPENTER. Phosphate exchange as the mechanism for adsorption of the radioactive isotope by the calcified tissues	713
GOLDWATER, WILLIAM H., and STETTEN, DEWITT, JR. Studies in fetal metabolism	723
HORN, MILLARD J., JONES, D. BREESE, and BLUM, AMOS E. Microbiological determination of threonine in proteins and foods	739
MILLER, GAIL LORENZ. Sedimentation, viscosity, and electrophoretic studies on purified Lee influenza virus preparations	745

Letters to the Editors

MACKENZIE, COSMO G., CHANDLER, JOSEPH P., KELLER, ELIZABETH B., RACHELE, JULIAN R., CROSS, NANCY, MELVILLE, DONALD B., and DU VIGNEAUD, VINCENT. The demonstration of the oxidation <i>in vivo</i> of the methyl group of methionine	757
EHRENSVÄRD, G., SPERBER, E., SALUSTE, E., REIO, L., and STJERNHOLM, R. Metabolic connection between proline and glycine in the amino acid utilization of <i>Torulopsis utilis</i>	759
AXELROD, A. E., HOFMANN, KLAUS, and DAUBERT, B. F. The biotin activity of a vaccenic acid fraction	761
FRIEDBERG, FELIX, WINNICK, THEODORE, and GREENBERG, DAVID M. Peptide synthesis <i>in vivo</i>	763
BLOCH, KONRAD, and ANKER, H. S. Synthesis of glutathione in isolated liver	765
INDEX TO VOLUME 169	767

THE EFFECT OF ADRENALECTOMY, ADRENAL CORTICAL HORMONES, AND TESTOSTERONE PROPIONATE PLUS ADRENAL CORTICAL EXTRACT ON THE ARGINASE ACTIVITY OF THE LIVER AND KIDNEY OF THE RAT*

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There are, among the steroid hormones, compounds which stimulate protein anabolism, the "N" hormones¹ (4, 1, 2), and protein catabolism, the "S" hormones¹ (glyconeogenesis from protein in the fasting organism) (5, 6). It is conceivable that these two groups of hormones may play a rôle in the balance of certain phases of protein metabolism in the body (*cf.* (1)).

It has been suggested that the action of the S hormones is mediated through the liver arginase, which is increased in chronically treated adrenalectomized and hypophysectomized but not in normal rats (7). Testosterone propionate, on the other hand, does not decrease this enzyme of the liver either in normal² (7) or castrated² rats and castrated mice (8), guinea pigs,² or hamsters.² It seemed, however, that the increase in protein catabolism and liver arginase induced by the adrenal cortical hormones could be at least partly prevented by previous treatment of rats with a potent N hormone, *e.g.* testosterone propionate.

Method

Adrenalectomy—White rats of the Wistar strain were adrenalectomized under ether anesthesia in groups of eight to twelve.

Hormone Preparations—2.5 mg. of testosterone propionate,³ in 0.1 ml. of sesame oil, were injected in the morning and the evening from the day of operation, including the morning of the day of the autopsy.

* This investigation was aided by grants from the Josiah Macy, Jr., Foundation and the Ciba Pharmaceutical Products, Inc.

† Part of the data in this paper was taken from a thesis submitted by Virginia N. Vail to the Graduate School, The University of Rochester, in partial fulfillment of the requirements for the M.S. degree, July, 1945.

¹ The terminology of Albright (1) is used, especially since there is no assurance that the nitrogen-retaining properties of the steroid hormones parallel their androgenic activity (*cf.* (2, 3)).

² Unpublished work.

³ The testosterone propionate (perandren) and desoxycorticosterone acetate (percorten) were provided by the Ciba Pharmaceutical Products, Inc.

The desoxycorticosterone acetate³ was injected at 1 mg. per day from the day of operation.

TABLE I

Effect of Adrenalectomy, Adrenal Cortical Hormones, and Testosterone Propionate (TP) Plus Adrenal Cortical Extract on Arginase Activity of Liver and Kidney of the Rat

Treatment*	Body weight gm.	No. of rats	Urinary nitrogen during injection period		No. of rats	Arginase			
						Liver		Kidney	
						units per gm.	per cent	units per gm.	per cent
Normal	145	7	192 ± 14†	+53	7	9800 ± 2060‡	+86	137 ± 27‡	+37
Control	156	8	126 ± 27		10	5260 ± 1140		100 ± 22	
Water <i>ad libitum</i>	144	3	148 ± 55	+18	8	4860 ± 625	-8	106 ± 15	+6
DCA 1 mg. per day	161	4	141 ± 33	+12	6	4900 ± 1930	-7	121 ± 27	+21
ACE§ 8 × 0.5 ml. per hr.	157	7	212 ± 26	+68	13	5130 ± 1620	-2	124 ± 30	+24
ACE§ 9 × 1.0 ml. per hr.	151	3	214 ± 25	+70	11	5460 ± 1120	+4	122 ± 32	+22
ACE§ 8 × 2.0 ml. per hr.	142	6	257 ± 39	+104	6	4890 ± 1020	-7	111 ± 25	+11
TP 2 × 2.5 mg. per day + ACE§ 8 × 0.5 ml. per hr.	158	6	204 ± 28	+62	11	6310 ± 1270	+20	197 ± 10	+97
TP 2 × 2.5 mg. per day + ACE§ 9 × 1.0 ml. per hr.	163				10	3870 ± 860	-26	213 ± 26	+13
TP 2 × 2.5 mg. per day + ACE§ 8 × 2.0 ml. per hr.	150	6	290 ± 66	+130	6	5120 ± 1060	-3	230 ± 37	+130

* All of the rats (except normals) were adrenalectomized and given 1 per cent sodium chloride as drinking water, except as indicated (water *ad libitum*) and the rats which received desoxycorticosterone acetate (DCA).

† Per cent change from adrenalectomized controls receiving 1 per cent sodium chloride as drinking water

‡ Standard deviation = $\sqrt{\Sigma d^2 / (n - 1)}$.

§ Adrenal cortical extract (ACE) (aqueous, Upjohn) was injected at hourly intervals on the last day

The adrenal cortex extract (aqueous, Upjohn⁴) was concentrated just before use *in vacuo* at 30-40° to about one-half its volume in order to re-

⁴ Part of this material was provided by Dr. M. Kuizenga, The Upjohn Company, and the rest was purchased from local dealers.

move the alcohol; the volume was restored with water, except in the 2 ml. per injection experiments, when it was made to exactly one-half of its original volume. The injections were given on the 5th postoperative day at hourly intervals for 8 hours (9), except in the case of one group which received nine injections (cf. Table I).

Urinary Nitrogen—The rats were placed in metabolism cages as a rule 3 days before the operation and were given 12 gm. of Purina fox chow meal and 1 per cent sodium chloride or tap water (cf. Table I) at 5 p.m. each day. The urine was collected daily just before feeding. On the 4th postoperative day no food was given; a urine collection was made on the following morning just before the injections of the adrenal cortical extract were begun, at which time the drinking tubes were removed from the cage (10), and a final collection was made at autopsy. The urine in the bladder was added to the last collection.

The total nitrogen content of the various collections was determined by the micro-Kjeldahl procedure.

Preparation of Tissue Homogenates and Enzyme Determination 1 hour after the last injection of the adrenal cortical extract the rats were injected intraperitoneally with sodium amytal at 9 mg. per 100 gm. of body weight. The left section of the median lobe of the liver⁵ and the right kidney were removed, weighed on a Roller-Smith torsion balance, and placed in separate heavy walled Pyrex tubes containing 5 ml. of cold redistilled water. The tissue samples were homogenized (8, 11), and enough water was added to give a concentration of 20 ml. of water per gm. of tissue. The liver homogenate was diluted further by pipetting 1 ml. into a 100 ml. volumetric flask containing 10 ml. of sodium barbital buffer,⁶ pH 9.4, and making to volume with redistilled water (8). The diluted material was filtered through two layers of cheese-cloth to remove large particles of connective tissue. The arginase was determined as previously described (8, 11).

Results

Urinary Nitrogen—The untreated adrenalectomized rats excreted less nitrogen in the urine during the period of fasting than the normal rats. The administration of 1 per cent sodium chloride as drinking water or 1 mg. per day of desoxycorticosterone acetate did not restore the nitrogen excretion to normal. The intensive treatment with adrenal cortical extract, on the other hand, increased the nitrogen excretion up to and above

⁵ The remainder of the liver was analyzed for glycogen, which was greatly increased by the adrenal cortical extract (Vail and Kochakian, unpublished)

⁶ If the sodium barbital buffer is omitted, there is a rapid and marked decrease in activity (Kochakian, unpublished). This decrease can be prevented also by the addition of cobaltous chloride (12, 13).

normal. The previous treatment with testosterone propionate had no significant effect on nitrogen excretion of the rats treated with the adrenal cortical extract.

Arginase—The arginase activity of the liver of the untreated adrenalectomized rats was approximately one-half that of the normal rats. None of the various treatments significantly alleviated the effects of the adrenalectomy.

The arginase activity of the kidney also was decreased after adrenalectomy but not as greatly as that of the liver. Furthermore, both desoxycorticosterone acetate and the various doses of the adrenal cortical extract partly, but not entirely, alleviated the effect of the removal of the adrenals. The administration of testosterone propionate, on the other hand, increased the enzyme content to slightly more than twice that present in the kidneys of the adrenalectomized rats.

DISCUSSION

The decrease in urine nitrogen excretion by the fasting adrenalectomized rat and the increase on treatment with adrenal cortical extract or certain of the C₁₁ cortical steroids are now well known (5, 6).

The inability of previous treatment with testosterone propionate to reduce the marked protein-catabolic effect of the adrenal cortical extract indicates that there are no antagonistic or competitive effects between the N and the S hormones in acute experiments of the type employed in this study. It must be borne in mind, however, that the intensive treatment of the rats with the adrenal cortical extract may have "overwhelmed" the previously established protein-anabolic effect of testosterone propionate (2). It is possible that under milder conditions a counterbalancing effect between these two types of protein hormones may exist.

The decrease in liver arginase after adrenalectomy is in agreement with a previous (7) and a recent (14) report. The failure of desoxycorticosterone acetate to affect the enzyme confirms the observation of Fraenkel-Conrat *et al.* (7). This hormone, however, effectively increases the enzyme when administered at a dose level of 2 mg. per day (7) and 3 mg. per day (14). Furthermore, the latter dose has been shown to be the most satisfactory for the maintenance of lactation in adrenalectomized rats (*cf.* (14)).

Contrary to expectation (7), the adrenal cortical extract did not restore the liver arginase to or even toward normal. This is especially remarkable since the extract produced a marked increase in nitrogen excretion, accompanied by marked increase in liver glycogen. Therefore, an increase in glyconeogenesis from protein under S hormone stimulation does not require an increase in liver arginase. The difference between these results and those of Fraenkel-Conrat *et al.* (7) and Folley and Greenbaum (14) is due

to the conditions of the respective studies. The increases noted by these investigators were obtained after treatment over a period of days. Furthermore, no remarkable effects were obtained in normal rats (7). Thus their results may be interpreted as a reflection of the partial restoration of the animals to a normal state, an anabolic rather than the catabolic effect suggested by Fraenkel-Conrat *et al.* (7). Indeed, 17-hydroxy-11-dehydrocorticosterone, a very potent S hormone, has been demonstrated to prolong the survival and increase the body weight of immature adrenalectomized male rats (15).

It is of interest that, after adrenalectomy, the liver loses much of its arginase activity but retains its full activity to deaminize alanine and glutamic acid (16). The kidney, on the other hand, not only loses a great deal of its arginase activity but also its ability to deaminize glutamic acid (16) and alanine (17, 16).

Both desoxycorticosterone acetate and the adrenal cortical extract were somewhat effective in restoring the kidney arginase toward normal, but the substitution of 1 per cent sodium chloride as drinking water was ineffective. It seems, therefore, that the kidney arginase-restoring ability of the adrenal cortical hormones is not associated with their electrolyte-regulating property.

The very marked increase in the kidney arginase of the rats previously treated with testosterone propionate is due in all probability to this compound *per se*, for similar increases have been noted in castrated rats injected with testosterone propionate (*cf.* (3)). It is noteworthy that these great changes are produced in such a short period of time and in the absence of the adrenal cortex.

SUMMARY

Adrenalectomy decreased the fasting urinary nitrogen excretion and the arginase activity of the liver and kidney of young ($150 \pm \text{gm.}$) adult male rats. The administration of 1 per cent sodium chloride as drinking water was ineffective and desoxycorticosterone acetate, 1 mg. per day, had a slight alleviating effect on only the decrease in kidney arginase. The administration of adrenal cortical extract (aqueous, Upjohn) at hourly intervals for 8 hours on the 5th postoperative day greatly increased the urinary nitrogen but did not affect the liver arginase, and partly restored the kidney arginase. Previous treatment with testosterone propionate, 2.5 mg. twice per day, did not alter the effects of the adrenal cortical extract but greatly increased the kidney arginase.

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β -GLUCURONIDASE: ITS RELATION TO THE ACTION OF THE ESTROGENIC HORMONES

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The increase in β -glucuronidase activity in the uterus of ovariectomized mice which follows estrogen injection (1) indicated that this enzyme was involved in the synthesis of β -glucuronides by the uterus. The increase in enzyme concentration which follows the administration of drugs which are excreted as glucuronides (2, 3) was similarly interpreted. It is important to note that there is a marked specificity exhibited in the site of action of these various agents, *i.e.*, the estrogens increase the enzymic activity of the uterus without appreciable alteration of the liver enzyme, while the glucuronidogenic drugs increase the enzyme in the liver, kidney, and spleen and are without effect on the concentration of enzyme in the uterus. It seemed reasonable therefore to believe that β -glucuronidase participated in the tissue transport or metabolism of the estrogens, and the experiments described in this report were designed to test this hypothesis. To this end mice were treated with various estrogens natural and artificial, and the enzyme concentration in different tissues was studied. Following estrogen injection, an increase in enzymic activity was found in the uterus and not in several other tissues. The effective dose of estrogen was very small, well within physiological limits, so that the response obtained appears to be a normal tissue reaction rather than an attempt upon the part of the organism to deal with a foreign toxic agent by conjugation and elimination.

Study of Enzyme in Various Tissues following Injection of Estrogenic Hormones

Ovariectomized mice were injected with aqueous alcoholic solutions of estrone, estriol, estradiol, estriol glucuronide, and stilbestrol, as previously described (1). However, instead of individual organ determination, assay of the enzyme was performed on a homogenate of pooled organs of each experimental group of three mice (3). Blood was collected from the chest cavity, oxalated, and separated into cells and plasma. The cells were washed with saline and laked. Glucuronidase determinations were performed on the liver, kidney, spleen, uterus, vagina, laked blood cells, and plasma of each group of mice. The results are given in Table I.

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Of the tissues listed, with the exception of uterus and in some cases vagina, which show elevated activities, the enzyme activity of the liver, kidney, spleen, blood cells, and plasma remains largely unchanged, sub-

TABLE I
Effect of Estrogens on β -Glucuronidase Activity of Various Tissues of Ovariectomized Mouse

Experiment No.	Estrogen (in aqueous alcohol)	Total dosage	β -Glucuronidase activity*						
			Liver	Kidney	Spleen	Uterus	Vagina	Blood cells per ml.	Blood plasma per ml.
1	Controls	0	3.18	1.31	6.64	0.92	1.03	0.24	0.08
	Estrone	0.5	4.42	1.85	6.95	2.90	2.36	0.26	0.05
	Estradiol	0.5	4.23	1.40	7.31	3.22	1.68	0.31	0.07
	Estril	0.5	3.35	1.26	5.72	1.87	1.21	0.26	0.13
	"	72.0	4.15	1.35	6.22	2.70	1.47	0.28	0.08
2	Controls	0	2.28	1.56	3.89	0.89	0.94	0.37	0.16
	Estrone	72.0	2.32	1.36	4.66	3.08	2.67	0.26	0.09
	Estradiol	72.0	2.43	1.44	4.50	2.93	1.43	0.31	0.14
	Estril glucuronide	72.0	2.18	1.51	6.22	4.70	2.40	0.53	0.09
	Stilbestrol	72.0	2.16	1.35	3.90	3.28	1.33	0.26	0.11

The estrogen was injected subcutaneously in a total volume of 1.2 ml. of 8 per cent alcohol, divided into 0.2 ml. doses twice a day for 3 days. Control mice received 8 per cent alcohol alone. The animals were killed on the 5th day and aqueous homogenates of the pooled organs of each experimental group (three mice) were prepared. The system consisted of 0.40 ml. of tissue extract, 0.40 ml. of 0.1 N acetate buffer (pH 5.0), and 0.2 ml. of 0.15 N sodium menthol glucuronide. Incubated 70 hours at 37.5°. The glucuronic acid liberated is estimated from the increase in reducing power of the digest over that of a boiled enzyme control (3). The limits of variation which can be expected in any single determination may be stated in terms of percentage variation as follows: liver, ± 19 (sixty-three mice), kidney ± 25 (fifty-seven mice), spleen, ± 25 (fifty-four mice), uterus, ± 33 (thirty-nine mice), and vagina, ± 27 (thirty-four mice). Similar data on pooled organs under these exact experimental conditions have not been collected. However, in other experiments with a 22 hour period of incubation (Table II), data on pooled organs have shown limits of variation which are not greater certainly than those given for determinations on individual mice. It has proved to be more satisfactory to make comparisons by referring to the values of a control group, run simultaneously, rather than to a composite average. In Table I, the enzyme activities in the control groups are in the higher range of the normal.

* Mg. of glucuronic acid liberated per hour per gm. of wet tissue.

sequent to estrogen injection, compared to the corresponding control group. Similarly, when 2 γ of estradiol monobenzoate were administered in oil in three daily injections, the uterine enzymic activity was elevated, but that of liver, kidney, and spleen was definitely unaffected (Table II).

In these experiments it is interesting to note the similarity in the action of the artificial estrogen to the natural estrogens, and it may be noted that stilbestrol monoglucuronide has been isolated from the urine of rabbits injected with stilbestrol (4).

Another finding which seems significant is the presence of the enzyme in mouse blood. In humans,¹ both cells and plasma show β -glucuronidase activity.

Relation of Dosage of Estrogen to Glucuronidase Activity

Amounts of estriol, estriol glucuronide, estradiol, and estrone (0.025 to 36 γ) were administered to ovariectomized mice in groups of twelve, as previously described (1). Their uteri were analyzed individually and the averages for each group have been computed. The difference between this value and the value of the controls is expressed as a percentage increase. These values have been related to the dosage in Fig. 1.

It is evident that estrone, estradiol, and estriol are more effective at lower doses than is estriol glucuronide and that significant effects are obtained with amounts of estrogen in the physiological range. A similar result is reported in Table I. It may be significant that a plateau is reached with increasing amounts of estrogen.

Effect of Testosterone Propionate When Administered Simultaneously with Estradiol Benzoate

The purpose of this experiment was to determine whether or not the action of estrogen in elevating uterine glucuronidase could be prevented by androgen. From Table II, it is evident that, although the weight of the uterus is lower in the groups receiving estradiol benzoate and testosterone propionate simultaneously, the concentration of enzyme is still in the same range as in the group receiving estradiol benzoate alone. Similar results are obtained when the data are expressed on the basis of nitrogen content and when a longer period of observation is employed (Table III).

In these experiments somewhat different conditions have been employed (injection of esterified hormones in oil), so that a comparison of the effects of 100 γ of testosterone propionate with the estrogen series becomes difficult. Apparently 1 mg. of testosterone propionate will elevate uterine glucuronidase. The comparison on a more quantitative basis of the effects of androgens and other substances has been reserved for future study.

Whereas the ability of androgen to antagonize estrogen stimulation is wide-spread, its failure to do so in the case of uterine glucuronidase argues for a unique type of specificity of action by estrogen.

¹ Fishman, W. H., unpublished.

Expression of Uterine β -Glucuronidase Activity on Basis of Total Nitrogen

In tissues such as liver and kidney, whose composition is regulated within very narrow limits, it is permissible to express the enzyme concentration

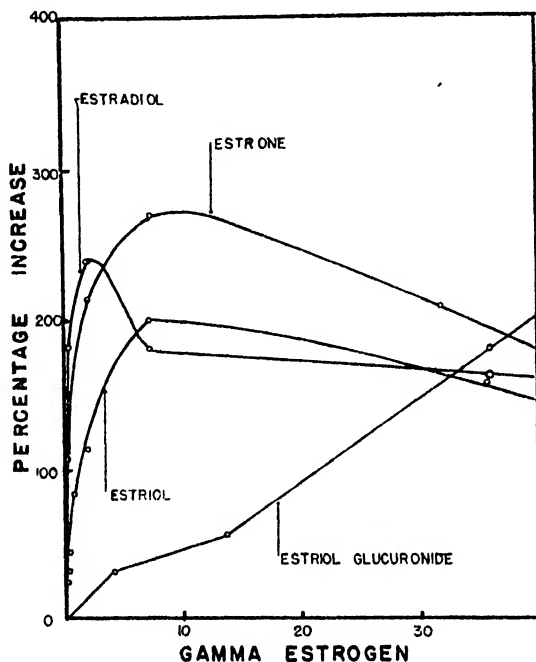


FIG. 1. The relation of dosage of estrogen to elevation in glucuronidase activity. The estrogen was injected subcutaneously in a total volume of 1.2 ml. of 8 per cent alcohol, divided into 0.2 ml. doses twice a day for 3 days. Control mice received 8 per cent alcohol alone. The animals were killed on the 5th day and aqueous homogenates of the uteri were prepared. The system consisted of 0.40 ml. of uterine extract, 0.40 ml. of 0.1 N acetate buffer (pH 5.0), and 0.20 ml. of 0.15 N sodium menthol glucuronide. Incubated 70 hours at 37.5°. The glucuronic acid liberated is estimated from the increase in reducing power of the digest over that of a boiled enzyme control (3). The difference in the enzymic activities in the estrogen-treated groups as compared to the controls is expressed as a percentage increase. The average uterine glucuronidase value for thirty-nine animals (individual analyses) in the control group was 0.68 ± 0.23 mg. of glucuronic acid per hour per gm. of wet tissue

on the basis of wet weight. However, in tissues such as uterus, whose water content is greatly augmented by estrogen administration, enzyme concentration on a wet weight basis is apt to be unsatisfactory.

The use of dry weight of tissue or total nitrogen as a basis for expressing

enzyme concentration in the uterus is advisable when a quantitative comparison of the effects of estrogen is being made. By this means the increase in enzymic activity is made more apparent, as in Table III and

TABLE II

Effect of Estrogen and Androgen on β -Glucuronidase Activity of Various Organs

Experiment No.	Substance administered (in oil)	Total dosage	Average uterine weight	β -Glucuronidase activity*			
				Liver	Kidney	Spleen	Uterus
		γ	mg.				
1	None	0	19.6	2.32	1.21	2.62	0.62
	Estradiol benzoate	2.0	140.0	2.29	0.87	3.15	1.30
	Testosterone propionate	100.0	44.0	2.13	0.96	2.43	0.98
	Estradiol benzoate + Testosterone propionate	2.0	71.5	2.76	0.90	2.60	1.72
		500.0					
	Estradiol benzoate + Testosterone propionate	2.0	64.0	2.95	1.13	2.98	1.50
2		1000.0					
	None	0	16.3	2.10			0.37
	Estradiol benzoate	1.0	83.5	2.74			1.32
	Testosterone propionate	1000.0	34.6	2.25			1.19
	Estradiol benzoate + Testosterone propionate	1.0	52.0	2.83			1.60
		1000.0					

The test substances were injected subcutaneously in a total volume of between 1.0 to 2.0 ml. of salad oil, divided equally into three daily doses. Control mice received salad oil alone. The animals were killed on the 4th day and aqueous homogenates of the pooled organs of each experimental group (three mice) were prepared. The system consisted of 0.40 ml. of tissue extract, 0.40 ml. of 0.1 N acetate buffer (pH 5.0), and 0.20 ml. of 0.15 N sodium menthol glucuronidate. Incubated 22 hours at 37.5°. The glucuronic acid liberated is estimated from the increase in reducing power of the digest over that of an average of two controls, one containing boiled extract in the presence of substrate and the other unboiled extract without substrate. The normal range of tissue enzyme activities obtained by duplicate analyses on six groups of ovariectomized mice (three mice to a group) was as follows: liver 1.83 to 2.88 mg. of glucuronic acid per hour per gm. of wet tissue, kidney, 0.72 to 1.70 mg., spleen 2.31 to 4.50 mg., and uterus 0.36 to 0.82 mg. From these data, the limits of variation which can be expected in any single determination on pooled organs can be stated tentatively in terms of percentage variation as follows: liver ± 11 per cent, kidney ± 29 per cent, spleen ± 16 per cent, and uterus ± 17 per cent.

* Mg. of glucuronic acid liberated per hour per gm. of wet tissue.

Fig. 2. Intrauterine fluid which accumulates in the estrogen-stimulated uteri often escapes when the uterus is removed. This factor, which would be an important source of variation and error in the determination of wet weight concentration, is of much less importance when the concentration is expressed on a nitrogen basis. In the previous experiments (2)

and in those reported in Tables I, II, and Fig. 1, it has not been necessary to make uterine nitrogen determinations, since the conclusions which are drawn are more of a qualitative than of a quantitative nature.

When 1 γ of estradiol monobenzoate is administered in oil, the total uterine nitrogen increases about 3-fold, while the enzyme concentration

TABLE III
Comparison of Enzyme Activities Based on Wet Weight and on Nitrogen Content of Uterus

Substance administered (in oil)	Dosage	Day following 3rd injection	Average uterine weight	Total activity*	Total N	Activity per mg. N	Activity per gm wet tissue
	γ		mg		mg.		
Control	0	0	19.7	0.09	0.76	0.12	0.45
Estradiol benzoate	1	1	71.2	1.18	1.30	0.91	1.66
" " +	1	2	31.9	0.59	0.69	0.85	1.85
Testosterone propionate	1000						
Estradiol benzoate +	1	5	44.5	0.58	0.94	0.62	1.30
Testosterone propionate	1000						

The test substances were injected subcutaneously in a total volume of between 1.0 and 2.0 ml. of salad oil, divided equally into three daily doses. Control mice received salad oil alone. These animals were killed on the 4th day and the estrogen-treated groups were continued for periods up to the 8th day of the experiment. Aqueous homogenates of the pooled organs of each experimental group (two mice) were prepared. The system consisted of 0.40 ml. of tissue extract, 0.40 ml. of 0.1 N acetate buffer (pH 5.0), and 0.20 ml. of 0.15 N sodium menthol glucuronidate. Incubated 22 hours at 37.5°. The glucuronic acid liberated is estimated from the increase in reducing power of the digest over that of an average of two controls, one containing boiled extract in the presence of substrate and the other unboiled extract without substrate. Uterine nitrogen was determined by micro-Kjeldahl analyses on suitable aliquots of the tissue homogenate. The normal range of uterine glucuronidase activities obtained by duplicate analyses on five groups of ovariectomized mice (three mice to a group) were as follows: 0.36 to 0.82 mg. of glucuronic acid per hour per gm. of wet tissue, and 0.12 to 0.22 mg. of glucuronic acid per hour per mg. of uterine nitrogen. From these data, the limits of variation which can be expected in any single determination on pooled uteri may be stated tentatively in terms of percentage variation as follows: uterus (wet tissue) ± 17 per cent, uterus (nitrogen) ± 23 per cent.

* Mg. of glucuronic acid liberated per hour

is elevated ten to thirteen times (Fig. 2). The prolonged effect here is probably due to the delayed absorption of estrogen from the oil and the slower rate of its destruction and elimination, since in other unpublished experiments with dilute alcoholic solutions of estrogens the elevation of enzyme is of shorter duration.

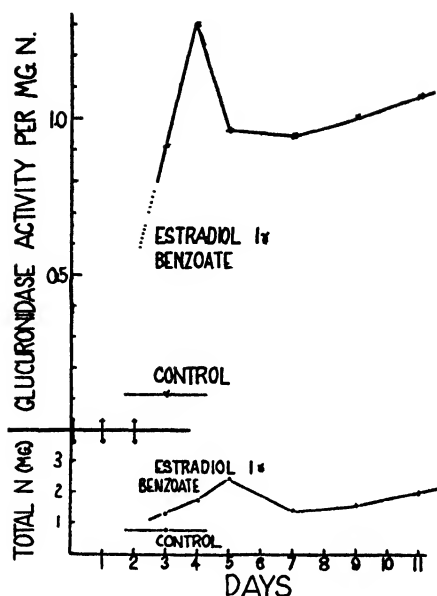


FIG. 2. A comparison of changes in uterine nitrogen and in glucuronidase concentration following the injection of estradiol benzoate. The system consisted of 0.40 ml. of tissue extract, 0.40 ml. of 0.1 *N* acetate buffer (pH 5.0), and 0.2 ml. of 0.15 *N* sodium menthol glucuronidate. Incubated 22 hours at 37.5°. The glucuronic acid liberated is estimated from the increase in reducing power of the digest over that of an average of two controls, one containing boiled extract in the presence of substrate and the other unboiled extract without substrate. Total uterine nitrogen was computed from micro-Kjeldahl determinations on suitable aliquots of the tissue homogenate. The normal range and limits of variation for the enzyme in the uterus of the ovariectomized mouse are given in the foot-note for Table III.

DISCUSSION

Glucuronide formation has generally been considered a process of "detoxication," since it has been observed most frequently in connection with the administration of drugs and other substances foreign to the body. However, this term, with its pharmacological connotation, cannot be applied properly to the formation of estriol and pregnanediol glucuronides in pregnancy. It is proposed that glucuronide formation in all cases be regarded as one of the processes of "metabolic conjugation;" other examples would include conjugation with glycine, sulfate, and acetate. The biological purpose of such reactions should be settled in each instance on the basis of the available evidence, and not arrived at by analogy.

β -Glucuronidase may be involved in metabolic conjugation processes with glucuronic acid as the conjugating substance. Thus Fishman (2, 3)

observed that glucuronidogenic substances, when fed to dogs and mice, induced an increase in the β -glucuronidase activity of various tissues. DeMeio and Arnolt found that the ability of liver and kidney slices to conjugate phenols was markedly increased when borneol or phenol was fed to rats (5). *In vitro* synthesis of glucuronides² from phenol (6) and borneol (7) in the presence of glucuronic acid has been observed with purified spleen glucuronidase (8) and with surviving liver slices (7, 6).

What is the nature of the elevation in the activity of glucuronidase, whether in liver following menthol feeding or in uterus subsequent to estrogen injection? Is the actual amount of enzyme increased or may there be involved the formation of an activator or the removal of inhibitor substances? If the actual amount of enzyme protein is increased, then this might possibly be an example of the adaptation of enzyme to substrate, as has been demonstrated for many microbial enzymes. It is true that the amount of uterine nitrogen is increased along with the marked elevation of uterine glucuronidase, but it is scarcely to be doubted that the greatest portion of the increment is the result of tissue growth. The proportion of enzymic to non-enzymic nitrogen is unknown and will be difficult to estimate. The differences in the response of the enzymes of liver, kidney, and spleen on the one hand and the enzyme of the uterus on the other may be due to actual differences in two enzymes or they may be explained by mechanical factors, such as permeability of the cell membrane to the stimulating agent.

It is important to decide to what extent the elevation in glucuronidase is a specific physiological response or merely a reflection of the increased growth of the uterus stimulated by estrogen. If the glucuronidase elevation phenomenon is connected with the amount of functioning protoplasm, it should be quantitatively related to the weight or, better, the nitrogen of the uterus and its concentration should not change. However, it is the *concentration* of the enzyme which is so markedly elevated, and from an inspection of Tables II and III and Fig. 2 it is clear that the amount of tissue *per se* is not the important factor which controls the glucuronidase response. Furthermore, the duration of the increase in glucuronidase concentration appears to be related to the duration of estrogen stimulation. This response is elicited with amounts of hormone within the physiological range. Accordingly, there seems to be sufficient evidence to warrant considering the elevation in uterine glucuronidase as a physiological response to estrogen.

The hypothesis may therefore be stated that the physiological significance

² According to a recent report (Crépy, O., *Compt. rend. Acad.*, **223**, 646 (1946); *Chem. Abstr.*, **41**, 2143 (1947)), surviving liver slices of rabbit and guinea pig *in vitro* conjugate estrone, estradiol, and estriol with glucuronic acid.

of the enzyme is the synthesis of the glucuronide of the hormone as the initial step in the utilization of the complex by the tissue. Metabolic conjugation of the estrogenic hormones may have far-reaching physiological and clinical implications. As pregnancy proceeds, the excretion of the glucuronides of estriol and pregnanediol becomes progressively greater. As currently interpreted, this is seen as a process by which the fetus and the body are protected from the deleterious effects of large quantities of free estrogen. In view of the concept that the liver is the site of inactivation of estrogens, one wonders why the uterus, which is the site of hormone action, should also be the site of its inactivation. From the evidence presented in this report the opinion can be advanced that glucuronidase has a fundamental rôle in the physiological action of the estrogenic hormones.

SUMMARY

When estrogens are administered in physiological amounts to ovariectomized mice, there follows an increase in β -glucuronidase activity of the uterus. This effect is not apparent in several other tissues studied, even with the administration of relatively large amounts of estrogen. Stilbestrol, an artificial estrogen, stimulates uterine glucuronidase in the same manner as the natural estrogens when given under the same conditions. Testosterone propionate does not antagonize the effects of estradiol benzoate in the experimental conditions employed. It is proposed that β -glucuronidase has a fundamental rôle in the physiological action of the estrogenic hormones and that glucuronide formation be considered a process of "metabolic conjugation" rather than detoxication.

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THE ASSAY OF ANIMAL TISSUES FOR RESPIRATORY ENZYMES

VI. FURTHER STUDIES ON OXIDATIVE PHOSPHORYLATION*

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Previous investigators of oxidative phosphorylation have not been concerned with any quantitative differences which might exist between normal and pathological tissues, and were therefore able to utilize extracts of tissues in their studies. It is probable that the preparation of such extracts involved the removal of relatively more of the energy-depleting enzymes than of the energy-mobilizing enzymes.¹ Although such manipulations greatly favor the demonstration of oxidative phosphorylation, they are not acceptable from the standpoint of a quantitative tissue assay because an unknown fraction of the enzymes is necessarily discarded. However, these earlier studies, particularly those of Ochoa (2, 3), established the components of the reaction mixture and the precautions which need to be taken, so that the demonstration of oxidative phosphorylation in kidney homogenates (4) was greatly facilitated. It was subsequently shown (5) that homogenates of a variety of tissues were capable of coupling the energy of oxidation with phosphorylation. Furthermore, the uptake of phosphate during short periods of incubation was proportional to the amounts of tissue employed. There was, therefore, a sound basis for attempting to develop an assay system for the study of oxidative phosphorylation in tissue homogenates. The chief disadvantage of the method as originally described (4) was the technical problem of fractionating the phosphorus of the reaction mixtures so that the "true" inorganic phosphate could be properly determined; although this was readily accomplished with considerable precision, the procedure was sufficiently tedious to discourage its widespread application. This difficulty has now been eliminated, since the new method for the determination of "true" inorganic phosphate recently published by Lowry and Lopez (6) is particularly well suited for following the course of phosphate uptake in the reaction mixtures which we employ. A second

* This work was aided by a grant from the Jonathan Bowman Fund for Cancer Research.

¹ Work in this laboratory by Dr. W. C. Schneider has shown, for example, that nuclei contain an appreciable fraction of the cellular ATPase and a negligible fraction of the succinic dehydrogenase and cytochrome oxidase, and that the nuclei are removed from homogenates by centrifuging at moderate speeds (1).

objection to the previous work was the use of fluoride. One of the goals of the present work has been the development of enzyme systems which could be coupled with energy-requiring syntheses, and since fluoride might affect some of these reactions adversely,² a system which could maintain adenosine triphosphate (ATP) in the absence of fluoride was to be desired. This also has been accomplished, although further improvements are to be expected. It is the purpose of the present paper to describe an improved method for the study of oxidative phosphorylation which combines the principles of the homogenate technique with the available facts on the enzymes of oxidation and phosphorylation.

EXPERIMENTAL

The basic system which we employ is one in which a very small quantity of homogenized tissue (20 to 30 mg. of wet weight) is placed in a reaction mixture which contains an oxidizable substrate (succinate, malate, oxalacetate, lactate, pyruvate, citrate, glutamate, or α -ketoglutarate), a complete hydrogen transport system (coenzyme, cytochrome *c*), and a hydrogen acceptor (oxygen), plus inorganic phosphate, a phosphate acceptor (creatine), and a phosphate carrier (ATP). The oxygen uptake is readily measured on a conventional Warburg apparatus and the phosphate uptake is measured by determining "true" inorganic phosphate in the reaction mixtures at zero time and after a period of incubation. In the previous work it was shown (5) that the inorganic phosphate which disappeared could be exactly accounted for as the increase in phosphocreatine if appropriate amounts of creatine transphosphorylase were added. However, in the various tissues studied the inorganic phosphate which disappeared was either greater or less than the phosphocreatine which was formed, depending upon the tissue used. In all cases except liver a substantial fraction of the bound phosphate was in the form of phosphocreatine, and thus the main analytical problem is to distinguish phosphocreatine from inorganic phosphate. In the Fiske-Subbarow method (8) the phosphocreatine is hydrolyzed too rapidly by the molybdic acid to be effectively distinguished from inorganic phosphate and the compounds had to be separated by precipitating the inorganic phosphate with calcium (4, 9).

In the Lowry-Lopez method for inorganic phosphate (6), the conditions established by Fiske and Subbarow (8, 9) have been modified in several respects. The pH was shifted from 0.65 to 4.0, the molybdate concentration was reduced from 0.25 to 0.10 per cent, and ascorbic acid (10) was used as the reducing agent. The data (6) on the effect of the molybdate con-

² For example, we have found in unpublished experiments that fluoride concentrations as low as 0.0005 M will prevent the ATP-catalyzed oxidation of octanoic acid (7).

centration show that a still lower molybdate concentration, 0.05 per cent, gives maximum color development with inorganic phosphate at 10 minutes and almost negligible rates of color development with phosphocreatine, acetyl phosphate, or ribose-1-phosphate. While the use of 0.10 per cent molybdate probably facilitates extrapolation to zero time to obtain the true inorganic phosphate, we prefer the lower concentration, since under our conditions the extrapolation is generally unnecessary, as will be shown. The exact procedure is given below.

Method for "True" Inorganic Phosphate In our previous work on oxidative phosphorylation (4, 5) the Warburg flasks were chilled, and to the contents (3.0 ml.) were added 2.0 ml. of 17.5 per cent cold trichloroacetic acid to give a final concentration of 7 per cent of the acid. This procedure was continued in the present work but an aliquot of 0.10 ml. of the acid protein-free filtrate has been analyzed directly for inorganic phosphate by the Lowry-Lopez method (6) as follows: An acetate buffer is prepared in such a manner that the sample of acid filtrate plus the buffer occupy a volume of 2.5 ml. and have a pH of 4.2. This buffer consists of 44 ml. of 0.5 M sodium acetate, 40 ml. of 1.0 M acetic acid, and water to give a volume of 180 ml. Colorimeter tubes 13×100 mm. containing 2.4 ml. of the buffer are prepared in advance, with a blank containing 2.5 ml. of buffer. Duplicate 0.10 ml. aliquots from the protein-free filtrates are added to the colorimeter tubes, followed by 0.25 ml. of freshly prepared 1 per cent ascorbic acid and 0.25 ml. of 0.5 per cent ammonium molybdate in 0.05 N H_2SO_4 . The latter reagents are also added to the blank. The molybdate is added at 30 second intervals and the tube contents are mixed by shaking. The color is allowed to develop 10 minutes and the optical density is measured at the chosen wave-length (6600 Å in the present work) on the photoelectric spectrophotometer (Cenco-Sheard) at the same time interval for each tube. In our case, the optical density multiplied by 1110 gives the micrograms of inorganic phosphate per Warburg flask.

Stability of Bound Phosphate - At two points in the analysis it is important to know the stability of the phosphate compounds formed by oxidative phosphorylation.

After the reaction in the Warburg flask has been stopped with trichloroacetic acid, there is necessarily a lapse of time while the tubes are being centrifuged and the samples are being pipetted, since in the usual experiment sixteen to eighteen flasks are used. It is thus important to know whether the bound phosphate is liberated by the trichloroacetic acid during this time. A number of preliminary trials showed no significant loss in bound phosphate in trichloroacetic acid in periods of from 2 to 3 hours at 0°. Experiments were therefore carried out for more extended periods of time and are reported in Table I. In order to demonstrate the excellent

reproducibility of the data, four flasks were set up with identical reaction mixtures and duplicates were treated with trichloroacetic acid at 0 and 20 minutes respectively. The reaction mixture contained exactly 310 γ of added inorganic phosphate, 166 γ of labile phosphate in the form of ATP, approximately 180 γ of phosphate in the form of DPN,³ plus the small amounts of phosphate contained in 30 mg. of fresh rat kidney. The analyses show that the flasks at zero time contained very little inorganic phosphate other than what was added as such. The figures varied from 328 to 341 γ per flask at this time, and the extraneous inorganic phosphate contributed by the tissue, the ATP, and the DPN amounted to only 18 to 31 γ per

TABLE I

Stability of Bound Phosphate in 7 Per Cent Trichloroacetic Acid Filtrate at 0°

Reaction mixture (see the text; succinate substrate) and incubation time (20 minutes) chosen to give approximately a 50 per cent disappearance of original inorganic phosphate. Data are reported in micrograms of "true" inorganic phosphate per flask, on duplicate flasks, with the modified Lowry-Lopez method. Each figure is the average of duplicate aliquots.

Experiment No.	Time in trichloro- acetic acid	Flask contents, 0 min.	Flask contents, 20 min.	$-\Delta P_{0-20}$
	<i>hrs.</i>	γP	γP	γP
1	0.5*	328, 332	171, 173	159, 157
1	2.5	333, 333	186, 190	147, 143
1	5.0	332, 335	184, 191	150, 143
2	4	341, 338	179, 175	161, 165
2	22	334, 339	190, 191	147, 146

* This was the shortest time which the manipulations permitted.

flask. The figures did not increase significantly when the trichloroacetic acid filtrates were allowed to stand at 0° for various periods of time up to 22 hours, from which we may conclude that neither the ATP nor the DPN yields inorganic phosphate under these conditions. On the other hand, the inorganic phosphate in the filtrates from the incubated flasks increased slightly, due to the breakdown of the bound phosphate, when the filtrates were stored. The increase was scarcely significant at 5 hours, but amounted to a decrease in ΔP of approximately 10 per cent at 22 hours. Since at least half of the bound phosphate is in the form of phosphocreatine (see below), the rate of phosphocreatine breakdown is only about 1 per cent per hour, if one assumes that the remainder of the bound phosphate is stable. At any rate the total bound phosphate is sufficiently stable in the

³ Diphosphopyridine nucleotide, coenzyme I.

trichloroacetic acid filtrates at 0° to permit all the necessary manipulations as well as interruptions of several hours.⁴

The second point at which we are concerned with the stability of the bound phosphate appears during the actual development of the color after the molybdate has been added. In the Fiske-Subbarow method phosphocreatine breaks down during the course of 20 to 30 minutes in the acid molybdate. It was therefore of interest to compare the rates of color development in the two methods, with samples from flasks which had been incubated 0 and 20 minutes, as in Table I. The data are given in Table II, and demonstrate two points. The data obtained with the Lowry-Lopez

TABLE II

Stability of Bound Phosphate in Presence of Molybdate at 25°

Samples taken from the trichloroacetic acid filtrates used in Table I and analyzed by the Lowry-Lopez and Fiske-Subbarow methods. Optical density was determined at intervals after molybdate addition.

Method of analysis	Interval after molybdate addition	Flask contents, 0 min.	Flask contents, 20 min.
	<i>min.</i>	<i>log I₀/I</i>	<i>log I₀/I</i>
Lowry-Lopez	12	0.296, 0.300	0.154, 0.156
	24	0.300, 0.302	0.162, 0.167
Fiske-Subbarow	30	0.314, 0.322	0.246, 0.230
	42	0.314, 0.328	0.253, 0.235

method show that in the flasks taken at zero time the color reached maximum by 12 minutes and remained essentially constant, while in the case of the flasks taken at 20 minutes the color continued to increase slowly, indicating a very slow breakdown of phosphocreatine. It is clear that for most experiments a single reading at 10 minutes is adequate, while an extrapolation to zero time, as recommended by Lowry and Lopez, can easily be made if necessary. In the case of the data obtained with the Fiske-Subbarow method the color development had reached its maximum in 30 minutes and remained essentially constant. In this instance, however,

⁴ The previous statements on the stability of phosphocreatine in trichloroacetic acid filtrates have been conflicting. Irving and Wells (11) reported that in ice-cold 8 per cent trichloroacetic acid muscle filtrates no decrease of the labile compound occurred in 5 hours. Fiske and Subbarow (9) reported that the labile compound is essentially all phosphocreatine and remarked that "The statement recently made by Irving and Wells that no loss of labile phosphorus occurs in 5 hours at 0° is not correct." They apparently did not report any data on the point, although they did state that the rate of hydrolysis in muscle filtrates was initially 25 per cent an hour at room temperature.

the $-\Delta P$ value was only about one-half as great as the analyses by the Lowry-Lopez method. The difference indicated that not all of the esterified phosphate was in the form of phosphocreatine, since the Fiske-Subbarow method as used determined the sum of inorganic phosphate plus phosphocreatine.

Further evidence on this point was obtained by using both methods to determine the ΔP when creatine was present or absent from the original reaction mixture. The data are given in Table III. It was found that the presence of creatine gave no increase in the ΔP value when measured by the Fiske-Subbarow method, whereas the esterified phosphate increased by 63 γ when determined by the Lowry-Lopez method. The difference between the ΔP values by the two methods when creatine was not added may

TABLE III

Effect of Creatine on Phosphate Uptake

Samples taken from trichloroacetic acid filtrates from enzyme experiments carried out with and without creatine. Reaction components as in (5) with kidney and succinate. Comparison of phosphate methods. Each figure is the average of two flasks.

Method of analysis	Creatine addition	Flask contents, 0 min.	Flask contents, 20 min	$-\Delta P_{0-20}$
		γP	γP	γP
Lowry-Lopez (direct)	None	332	280	52
	30		217	115
Fiske-Subbarow (direct)	None	326	294	32
	30		294	32

be due in part to endogenous phosphate acceptors with esters labile to the Fiske-Subbarow procedure.

Further Comparison of Phosphate Methods—As a final proof of the validity of the new method, data obtained by the direct analysis of trichloroacetic acid filtrates by the Lowry-Lopez method were compared with data obtained on the same filtrates with the Fiske-Subbarow method, following fractionation by calcium precipitation, as in the earlier work (4, 9). The data are shown in Table IV. It is clear that as far as the disappearance of inorganic phosphate was concerned the two methods yielded results that were practically identical. The absolute difference between the two methods was only 10 γ in 330 γ and this may represent a loss of phosphate in the additional manipulations of the indirect Fiske-Subbarow method. For most work, the measurement of the ΔP by means of the direct Lowry-Lopez method is obviously the method of choice. For more detailed information the calcium fractionation can be used as previously described (4).

Components of Reaction Mixture—As a result of the previous work (4) and subsequent experience, the general principle of using low substrate concentrations has been established. It appears that the substrate concentration should be lower than the amount required to saturate the oxidative system. This is in contrast to the work on the oxidative systems alone, in which very high levels of succinate (12) or malate (13) were employed. In the cases cited, the final substrate concentration was 0.05 M. In the

TABLE IV

Comparison of Methods for Determination of "True" Inorganic Phosphate

The six experiments were run with the reaction media varied in such a way that the incubated flasks would contain inorganic phosphate and phosphocreatine in varying proportions within the range of values usually encountered. Each experiment was carried out in duplicate and the same enzyme preparation was used throughout. All of the flasks theoretically had identical amounts of inorganic phosphate at zero time and the variation among the zero flasks represents the experimental error of each method. The Lowry-Lopez method was employed directly, as described in the text, while the Fiske-Subbarow method was applied to the calcium precipitates after separation from phosphocreatine (4, 9). The analyses by the Fiske-Subbarow method were kindly performed by Mr. Harlan L. Klug. See the text.

Experiment No.	True inorganic phosphate per flask				-ΔP ₀₋₂₀	
	At 0 min.		After 20 min. incubation		Lowry-Lopez	Fiske-Subbarow
	Lowry-Lopez	Fiske-Subbarow	Lowry-Lopez	Fiske-Subbarow		
	γ	γ	γ	γ	γ	γ
1	334	328	205	203	135	127
2	343	334	286	277	54	53
3	336	328	232	224	108	106
4	343	326	231	230	109	100
5	346	335	243	236	97	94
6	340	331	300	288	40	42
Average. ...	340	330				

oxidative phosphorylation studies, the amount of the oxidizable substrate has been 4×10^{-3} M or less. The reason that the low substrate concentration yields better net phosphate uptake is believed to be the occurrence of general reactions of the type as previously suggested (4, 5). This concept

- (1) $\text{ATP} + \text{oxidizable substrate} \rightleftharpoons \text{ADP} + \text{phosphorylated substrate}$
- (2) $\text{Phosphorylated substrate} \rightarrow \text{oxidizable substrate} + \text{inorganic phosphate}$

has also been used by Utter and Wood (14) in connection with their experiments on CO₂ fixation. In their work the "oxidizable substrate" in

reactions (1) and (2) above was pyruvate. We have data to indicate that this is a general reaction which results in a "leak" of high energy phosphate from the ATP reservoir back to the inorganic level, and that the extent of the leak depends upon the nature and quantity of the substrate present.

The reaction mixture previously developed (4) has been changed slightly and the present system is reported here. The complete reaction mixture consists of the following components, *which are added in the order given*: 30 mg. of dry creatine hydrate, water to make a final volume of 3.0 ml., 0.20 ml. of 0.5 M KCl, 0.30 ml. of 0.10 M $MgCl_2$, 0.20 ml. of 0.133 M Na ATP, pH 7.5, 0.30 ml. of M/30 sodium phosphate, pH 7.5, standardized to contain exactly 310 γ of P, 0.10 ml. of 4×10^{-4} M cytochrome c, 0.30 ml. of 0.04 M sodium succinate, pH 7.5, 0.1 to 0.60 ml. of any additional cofactor or inhibitor under study, 0.20 ml. of 0.20 M NaF, and 0.20 or 0.30 ml. of a freshly prepared 10 per cent water homogenate of rat kidney. The center cup contains 0.2 ml. of 2 N NaOH plus filter paper. Precautions to insure speed and cold (4) are followed. The flasks are placed in cracked ice just before the homogenate is prepared, and the reaction is begun by transferring the flasks from the ice to the 38° bath. With malate and lactate the complete reaction mixture includes 0.1 ml. of a 1 per cent solution of DPN³ (about 60 per cent assay) at pH 7.2. The following section deals with the fluoride addition.

Precautions in Use of Fluoride—In the course of some work on inhibitors it was noted that the extent of "inhibition" was correlated with the volume of inhibitor solution. This was traced to the incompatibility of the Mg and the fluoride. It was found that, when the fluoride addition was delayed until just before the addition of the homogenate, no precipitation occurred, but that when the fluoride was added earlier it formed a precipitate with the Mg^{++} . This precipitate did not redissolve when the volume reached 3.0 ml. This point is so important that the quantitative aspect of the effect is reported in Table V. It is clear that the later addition of fluoride is mandatory and that the water should be added prior to the fluoride, as suggested in the preceding section. The amounts of fluoride and magnesium to be added are interrelated, and the amounts recommended in the preceding section are based upon experiments in which both were varied. As shown in Table V, the use of the lower level of fluoride made the artifact negligible at certain concentrations but more pronounced at others. The fact that a second portion of as much as 1.0 ml. of water could be added without altering the result, provided that the fluoride was added last, shows that variations in the volume of the other components or inhibitors can be safely carried out within this range.

Precautions against Use of Calcium—Ochoa (2) had suggested that the beneficial effect of fluoride in improving the net yield of esterified phosphate

might be due to the precipitation of calcium ions, which activate adenosine-triphosphatase (ATPase) in a variety of tissues (15). Since magnesium and fluoride ions can coexist in the reaction mixture at a much higher concentration than the solubility of MgF_2 would indicate, it was of interest to determine what the effect of added calcium would be. This type of experiment forms the prototype of tests of various inhibitors, and is reported graphically in Fig. 1. In this figure, the calcium concentration is stated in mg. per 100 ml. as well as molarity to facilitate comparison with the

TABLE V
Precautions in Use of Fluoride

The usual reaction mixture (see the section on "Components of reaction mixture") was employed, but, instead of the addition of the reactants in the order given, the $MgCl_2$ and the NaF were allowed to mix in fluid volumes varying from 0.9 to 2.8 ml by altering the order of adding the reactants. The order of adding the reactants was as given in the text, except that the water was added in two portions, with the second portion added to simulate the addition of an inhibitor. The fluoride was added either after the $MgCl_2$ or after the second water addition. The final volume was 3.0 ml. in all cases. The results are reported as micrograms of inorganic phosphate uptake per flask.

Fluoride addition	Inorganic phosphate taken up. 2nd addition of water							
	0.0 ml		0.3 ml		0.6 ml		1.0 ml	
	γ	P ml.*	γ	P ml.*	γ	P ml.*	γ	P ml.*
0.2 ml. 0.25 M NaF added after $MgCl_2$ addition	78	1.9	55	1.6	48	1.3	30	0.9
0.1 ml fluoride added as above	74	1.9	68	1.6	73	1.3	8	0.9
0.2 ml. fluoride added after 2nd addition of water	77	2.8	72	2.8	72	2.8	72	2.8

* The figures represent the volume of fluid present when the fluoride was added to the mixture that included magnesium ions.

calcium content of blood serum. It is evident that concentrations one-tenth as high as the serum calcium are very deleterious, while much lower concentrations produce noticeable effects. In the case of the succinate system the decrease in phosphate uptake is almost completely accounted for by the increased ATP breakdown, as is shown by the "no substrate" control curve, but in the oxalacetate system the decrease in yield is very striking and is not explained by the rate of ATP breakdown. Furthermore, the oxidation of oxalacetate is decreased by the presence of calcium ions, in contrast to the oxidation of succinate, which was unaffected in this system. Previous work (4) indicated that succinate probably does not participate in reactions of the types shown by equations (1) and (2) to a

significant extent. The mechanism of the calcium effect in the oxalacetate system is unknown, but it may involve reactions of the type shown in equations (1) and (2) above. The increased decarboxylation of oxalacetic acid which is caused by calcium ions may be involved, but this explanation is weakened by the fact that the rate of oxygen uptake is but slightly diminished by decreases in the substrate concentration (4). Furthermore, a number of other inhibitors have been shown to inhibit the oxalacetate system much more than the succinate system (to be published). Regardless of the mechanism, it is clear that calcium ions should be eliminated

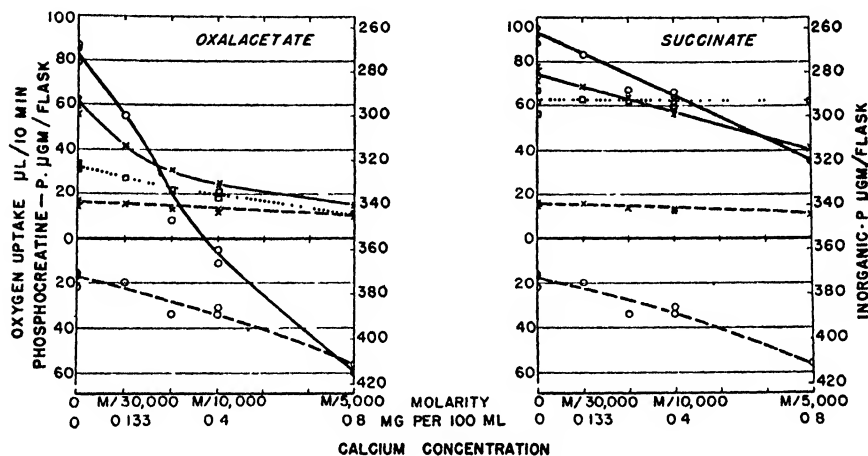


FIG. 1. The effect of calcium ions on oxidative phosphorylation obtained with water homogenates of rat kidney. The reaction mixture is described in the text; incubation time, 20 minutes. \circ (dash line), inorganic phosphate (no substrate); \times (dash line), phosphocreatine (no substrate); \circ (solid line), inorganic phosphate (substrate present, succinate or oxalacetate); \times (solid line), phosphocreatine (substrate present); \square , oxygen uptake (on succinate or oxalacetate).

from the reaction medium as well as from any inhibitor solutions which might be studied.

Summation of Oxidative Phosphorylations.—In a recent paper on the malic dehydrogenase system (13) it was shown that tissue homogenates require the addition of considerable amounts of DPN in order to obtain effective oxidation of malate. Combinations of malate, DPN, and succinate were therefore studied in the oxidative phosphorylation system, as is shown in Table VI. It was found that malate additions decreased the phosphate uptake caused by succinate oxidation, while the addition of DPN to the succinate system produced a marked increase in the phosphate uptake with but little increase in the oxygen uptake. It would appear that

the DPN stimulated the oxidation of malate formed from succinate and that increased amounts of malate were superfluous as far as the oxidation was concerned, and detrimental to phosphate uptake by some such mechanism as proposed in equations (1) and (2) above. In contrast to malate, lactate gave an increased rate of oxygen uptake and a slight increase in phosphate uptake. The explanation of the summation observed with lactate and succinate and the lack of summation observed with malate and

TABLE VI
Summation of Oxidative Phosphorylations

Each flask contained the reaction system described in the text, with 0.30 ml. of a 10 per cent water homogenate of rat kidney. The ΔP_{0-20} refers to the difference in inorganic phosphorus at 20 minutes compared with a zero control containing an identical reaction mixture.

DPN	Additions			ΔP_{0-20}	O ₂ per 10 min.
	Malate	Succinate	Lactate		
				γ	ml.
—	—	—	—	+14	9.3
+	—	—	—	—51	15.8
—	+	—	—	—55	29.6
—	—	+	—	—96	43.8
—	+	+	—	—74	40.1
+	+	—	—	—121	41.8
+	—	+	—	—179	49.6
+	+	+	—	—138	49.6
—	—	—	—	+15	4.6
+	—	—	—	—20	14.7
—	+	—	—	—60	30.1
—	—	+	—	—98	44.0
—	—	—	+	—24	18.0
+	+	—	—	—91	43.8
+	—	+	—	—137	49.0
+	—	—	+	—89	37.0
+	—	+	+	—146	60.6

succinate may be that malate is formed from succinate, while lactate is not so directly related. Thus, with succinate plus DPN there was probably summation between malate and succinate oxidation. The malate data reemphasize the importance of using a low substrate concentration.

Phosphorylation in Absence of Fluoride—The summation experiments suggested the possibility of attempting to maintain the ATP reservoir by means of a combination of oxidizable substrates. It seems probable that in a whole homogenate many of the enzymes which deplete the ATP reser-

voir may operate unrestrained by the mechanisms which control the use of ATP in the intact cell. It is unreasonable to expect a single channel of oxidative phosphorylation to keep pace with this unrestrained depletion, but by using a combination of oxidizable substrates one might expect to be able to bring into play a number of the oxidative systems which are capable of replenishing the ATP reservoir and thus to take up inorganic phosphate as rapidly as ATP is dephosphorylated. The present work dealt in terms of the components of the Krebs cycle. It was known from our previous study that in order to get effective phosphorylation most substrates have to be used at low concentrations (4), possibly because of reactions of the type shown in equation (1) above. It was also indicated that the carbonyl compound depleted the ATP reservoir to a greater extent than the corresponding hydroxy compound with the oxalacetate-malate pair of substrates. Potter and Schneider (unpublished) have also found that pyruvate and α -ketoglutarate tend to deplete the ATP reservoir. Since it seems desirable to add oxalacetate, pyruvate, and α -ketoglutarate in small amounts to avoid the "leak effect," and since they can be formed readily in the reaction mixture from malate, lactate, and glutamate, the latter compounds are more desirable additions for the present purpose. Since it was shown above (Table VI) that succinate plus DPN was superior to succinate plus malate plus DPN, succinate was used as a substrate and malate was omitted. Attempts to use citrate to complete the full utilization of the Krebs cycle by conversion to isocitrate were unsuccessful, and the final mixture of substrates included only succinate, lactate, and glutamate. The reactions set in motion by these additions are readily expressed in terms of the Krebs cycle (16). Glucose was added to provide an additional outlet for ATP and to provide, if possible, a continuing source of substrates for the Krebs cycle, but the data thus far have not indicated that the glucose is beneficial. Since one objective of this work is the reconstruction of the total system (see below), glucose was retained in the mixture. The nature of the data is such that the course of the experiment can best be pictured by plotting the change in the inorganic phosphate concentration with time. Data obtained with two different homogenates are shown in Fig. 2 to illustrate the precision of single experiments and the manner in which variation in the homogenates affects oxidation and phosphorylation. In each case, kidney was the tissue employed. Changes in the concentration of inorganic phosphate during a 30 minute incubation period are shown with four different reaction mixtures: three substrates present \pm fluoride (Curves GSF and GS) and three substrates omitted \pm fluoride (Curves GF and G). Thus there are four curves for each of two rats and a pair of curves for each of the four experimental set-ups, while each curve consists of data from four flasks containing identical re-

action mixtures with the reactions stopped at 0, 10, 20, and 30 minutes respectively by the addition of trichloroacetic acid. Each point on the phosphate curves represents duplicate analyses on a single flask, while the oxygen uptake data represent the average of the manometer readings on all the flasks up to the time that they were taken off for phosphate analysis. For each pair of curves, the upper curve (X) represents data from an older

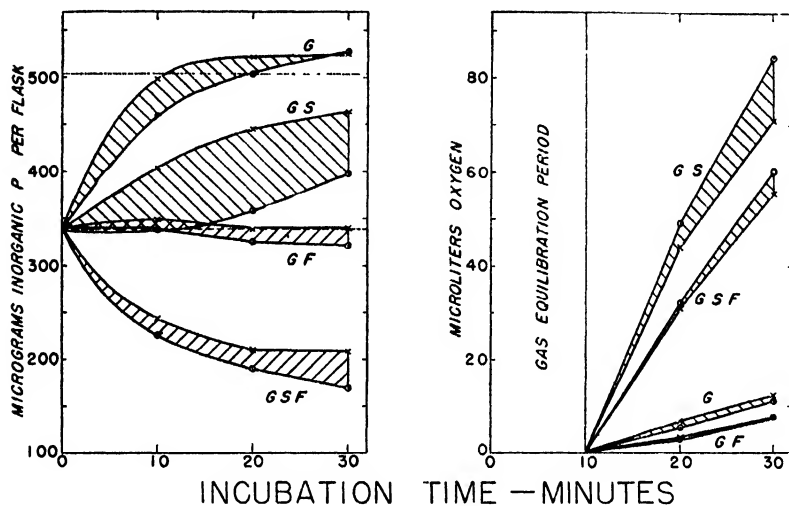


FIG. 2. The changes in inorganic phosphate concentration and the oxygen uptake obtained with 0.2 ml. of a 10 per cent water homogenate of rat kidney. The reaction mixture is as in the text, including DPN, but with variations in the substrate and fluoride as follows: Curves G, 6 mg. of glucose per flask; Curves GS, the same as Curves G but with 0.2 ml. of 0.2 M sodium fluoride, Curves GF, the same as Curves G but with succinate, lactate, and glutamate each added as 0.1 ml. of 0.04 M solution, Curves GSF, the same as Curves GS but with fluoride as in Curves GF. The dash line represents the original inorganic phosphate concentration in all flasks; the dotted line, the sum of the original inorganic phosphate plus the high energy phosphate of the added ATP. The curves represent single experiments with two different rats; 0 = rat 2 months old, X = rat 8 months old.

rat (8 months) and the lower curve (O) represents data from a younger rat (2 months). The significance of this consistent relationship lies in the fact that the curves are the result of enzyme systems which act in opposite directions with respect to ATP synthesis and breakdown, and the lower any curve falls the greater is the effect of the energy-mobilizing (phosphate-binding) enzymes, as compared to the effect of the energy-depleting (dephosphorylating) enzymes: The phosphate-binding system was relatively more active in the tissue from the younger rat in this instance. The oppos-

ing systems vary independently and the same plan of experiment is applicable to the study of accessory factors which improve the relative position of the phosphate-binding systems, or to the action of drugs or inhibitors which may increase the rate of phosphate breakdown relative to the rate of phosphate uptake.

In all of the reaction mixtures the variations did not affect the inorganic phosphate in the flasks taken at zero time and a horizontal broken line through the graph indicates this original level of inorganic phosphate (about 340 γ of phosphate per flask) as a base line for comparison with the changes produced by incubation. This line is based on actual determination of the inorganic phosphate levels in the eight experimental reaction mixtures. A second reference line is the dotted line at about the 505 γ level. This line is based on the sum of the actual original inorganic phosphate plus the theoretical amount of phosphate which could be liberated from the added ATP if both of the high energy phosphate groups were split off.

The data show that the rate of oxidation when glucose alone was present was very low in comparison to the rate attained when the three substrates of the Krebs cycle were added. When glucose and fluoride (Curves GF) were present, the oxygen uptake was very poor, but the ATPase was inhibited by the fluoride and the ATP was therefore not hydrolyzed; the inorganic phosphate concentration remained constant. When glucose was present without fluoride (Curves G) the oxygen uptake improved slightly, but the ATPase was unrestrained by the fluoride and the ATP was quickly hydrolyzed to the level corresponding to adenylic acid.

When the three substrates (lactate, glutamate, and succinate) were present in addition to glucose and fluoride (Curves GSF), vigorous oxygen uptake occurred and the inorganic phosphate level dropped rapidly with time as oxidative phosphorylation proceeded, with the ATP breakdown inhibited by fluoride. When fluoride was omitted (Curves GS), the oxidation rate was greater but the vigorous uptake of inorganic phosphate was opposed by a somewhat more vigorous ATP breakdown, since the ATPase was unrestrained in the absence of fluoride. This is the experimental set-up which is most sensitive to changes in the reaction mixture and it is this pair of curves which shows the greatest disparity between the two rats. Only in the case of the younger rat was it possible for the oxidative phosphorylation to keep pace with the ATP breakdown and to maintain the ATP reservoir, and even in this case the balance had definitely shifted in favor of the ATP breakdown within 20 minutes. This was correlated with a decreased rate of oxidation which may reflect an accumulation of keto acids. This reaction mixture is the best that we have been able to devise for the testing of reactions which simulate ATPase (*e.g.* reaction (1) + (2)) in the absence of fluoride. In this system, *the addition of sub-*

strates sufficient to saturate completely the oxidative mechanism results in a decreased yield of net phosphate uptake. This we interpret in terms of reactions (1) and (2), and since oxalacetate is more active in this respect than malate (4), any failure to metabolize the keto acids will cause their accumulation and will accelerate the "leak" of phosphate from ATP back to the inorganic phosphate level. The system can therefore be adapted to the search for the factors necessary for the oxidation of these keto acids or for the study of inhibitors of the transphosphorylases. It is clear that any compound which can substitute for "oxidizable substrate" in equation (1) by reacting with ATP to give a phosphorylated compound which can be readily hydrolyzed will have the effect of strongly "inhibiting" phosphate uptake by providing an auxiliary reaction which will simulate ATPase. Furthermore, any *functional* type of reaction in which ATP energy is utilized would simulate ATPase. For example, if acetate and choline were added to such a system in the presence of choline acetylase, an increased output of inorganic phosphate should result. If the functional enzymes can be prepared free from ATPase, the present reaction mixture can be by-passed and pure ATP substituted for the system which maintains it, but otherwise it is clear that the ATP will be depleted rapidly (Curves G). The problem is simplified if the functional enzyme is insensitive to fluoride, since the oxidative phosphorylation system is relatively insensitive to it; at the 10 minute point, Curves G minus Curves GS are approximately equal to Curves GF minus Curves GSF. Since it cannot be assumed that the functional enzymes will be insensitive to fluoride and since the preliminary work must in all likelihood be done with crude preparations containing ATPase, the development of systems capable of maintaining the ATP reservoir even for short periods of time (Curve GS, lower line) is an essential step in the development of reaction systems which utilize ATP energy for purposes of synthesis. Meanwhile, further improvements in the system should prolong the period during which the ATP reservoir can be maintained (see below).

Glycolytic Enzyme Complex—The data in Fig. 2 show that when glucose was the sole oxidizable substrate little oxidation or phosphate uptake occurred, in spite of the fact that ATP, DPN, and cytochrome *c* were present. Since glucose would have to be converted to hexose diphosphate (HDP) before being oxidized (assuming the hexose monophosphate shunt (16) to be inoperative in the absence of added triphosphopyridine nucleotide), the first addition to the system was HDP. Using reaction mixtures analogous to those for Curves G and GF in Fig. 2, we determined the effect of adding hexose diphosphate at a final concentration of 0.004 M. This resulted in a vigorous oxygen uptake which was only slightly inhibited by fluoride. In the presence of fluoride, phosphate uptake occurred only

during the first 10 minutes and then remained stationary. In the absence of fluoride the rate of inorganic phosphate output paralleled Curves G in Fig. 2 for the first 10 minutes and then continued at the same rate for the entire 30 minutes, instead of leveling off at the ATP plateau in 10 to 20 minutes. Thus HDP was vigorously dephosphorylated in the absence of fluoride. The results with HDP (Table VII) showed conclusively that the low rate of oxidation observed with glucose alone (Curve G, Fig. 2) was due to an inadequate conversion of glucose to HDP rather than to an inability to oxidize HDP. Excellent work on glycolysis in *brain* homogenates had been done by Utter, Reiner, and Wood (17, 18), who also found that HDP could not be supplanted by glucose plus ATP. They used anaerobic conditions and a bicarbonate buffer. Racker and Krimsky (19, 20) have also made important contributions to the study of glycolysis in brain homogenates, and the low metabolism of glucose as compared

TABLE VII
Oxidation of Glucose Compared with Hexose Diphosphate

Reaction mixture as indicated in Fig. 2, except that sodium hexose diphosphate at a final concentration of 0.004 M was added as indicated.

Addition	O ₂ uptake per 20 min	
	With fluoride	Without fluoride
	<i>microliters</i>	<i>microliters</i>
Glucose	9.4	9.2
" + hexose diphosphate	31.3	42.9

with HDP which was observed by us and by Utter *et al.* may be explained by the finding (19) that sodium ions inhibited the glycolysis of glucose but not the glycolysis of glucose-6-phosphate, fructose-6-phosphate, or HDP. Racker and Krimsky suggested that sodium ions might act by inhibiting the phosphorylation of glucose; yet the test systems employed for the assay of hexokinase have consisted of sodium salts (21, 22). The explanation is also complicated by the observation that phosphocreatine neutralized the sodium effect (19). A multiplicity of factors influencing the hexokinase reaction has been reported by the Cori group and include reduced DPN (23), guanine (24), and insulin (25). Whether these factors are limiting in a fresh homogenate remains to be seen.

For the present, we have circumvented the hexokinase reaction by using HDP as the starting point for glycolysis, which has been studied in terms of the oxygen uptake resulting from the oxidative step. Racker and Krimsky (19) had obtained the oxygen uptake in a similar system but had not added cytochrome *c* to complete the hydrogen transport system. We

observed marked stimulation of oxygen uptake by the addition of cytochrome *c* and found that the amount we had previously established for the Krebs oxidations was an appropriate level (Table VIII). Confirming the results of Racker and Krimsky, we found that acid production due to glycolysis quickly lowers the pH to below the optimum, and, like them, we have been able to control the pH by adding bicarbonate. It was

TABLE VIII

Effect of Cytochrome c on Hexose Diphosphate Oxidation

Conditions as in Table VII, except that 0.2 ml. of 0.1 M sodium bicarbonate was added and 0.3 ml. of kidney homogenate was used. Glucose, HDP, and fluoride were present. Phosphate and cytochrome *c* varied as shown. Oxygen uptake in 20 minutes

Addition, m/30 phosphate	1×10^{-4} M cytochrome <i>c</i> per flask		
	None	0.1 ml	0.4 ml
	microliters O_2	microliters O_2	microliters O_2
0.1	36.5	56.0	56.6
0.6	46.4	78.4	86.6

TABLE IX

Effect of Sodium Bicarbonate on Hexose Diphosphate Oxidation

Conditions as in Table VII, except as otherwise indicated. Glucose, HDP, and fluoride were present in all flasks.

Experiment No	Homo- genate	Phos- phate	0.1 M sodium bicarbonate per flask							
				0.1 ml	0.2 ml	0.3 ml	0.4 ml	0.5 ml	1.0 ml	
			ml.	ml	microliters O ₂	microliters O	microliters O ₂	microliters O ₂	microliters O ₂	microliters O ₂
1	0.3	0.1	35.1						75.3	65.2
2	0.3	0.6		71.5	86.6	91.6				
3	0.2	0.1			50.7	53.8	56.9			
3	0.2	0.3			57.3	58.8	57.8			
3	0.2	0.6			64.1	63.0	64.5			

found that when the pH was held at 7.3 to 7.6 by means of 0.2 to 0.4 ml. of 0.10 M bicarbonate, the maximum stimulation of oxygen uptake occurred (Table IX). The bicarbonate addition results in CO_2 evolution when acid is formed, and by empirically⁵ establishing the amount of bicar-

⁵ If acid is not formed, the reaction becomes alkaline, since the bicarbonate loses CO_2 to the center cup (26). The amount of bicarbonate must therefore be controlled for each experimental set-up. It is clear that the ability of bicarbonate to neutralize acid is extremely important in the "stimulation" of respiration (27) and glycolysis (28) by this substance.

bonate it is evidently possible to absorb the CO_2 in the center cups as rapidly as it is formed so that the pressure changes represent oxygen uptake. Other methods of controlling the pH as well as the factors involved in the phosphorylation of glucose are under study. However, with the present system with bicarbonate, the oxygen uptake on HDP is about equal to the oxygen uptake on the three substrates shown in Fig. 2. The use of nicotinamide to inhibit DPN destruction is very important in brain homogenates (17-20) but has little effect on kidney glycolysis, probably because kidney contains less DPNase (29).

DISCUSSION

The results in this paper and the two preceding papers (4, 5) establish the fact that it is possible to demonstrate oxidative phosphorylation in homogenates with a variety of tissues and substrates and provide a basis for the use of certain analytical methods, principles, and precautions. The system has been shown to be capable of maintaining the ATP reservoir for some 20 to 30 minutes in the absence of fluoride with homogenates of rat kidney, and may therefore be useful for the study of reactions which utilize ATP energy as well as for the study of the reactions which result in ATP synthesis. It is by no means certain that the optimum quantity of essential reactants is established, and possibly there are essential reactants of unknown identity.

Attempts have been made to coordinate this work with the studies by Utter *et al.* (17, 18) and by Racker and Krinsky (19, 20) so that the interplay of the glycolytic and oxidative systems can be studied. The level of inorganic phosphate which can be attained by the oxidation of the substrates in the Krebs cycle is quite low and definitely falls within the range which Warburg and Christian found to influence the oxidative step in glycolysis (30). The level of phosphate used in the other studies on glycolysis (17-20) was much higher than the levels used in this work and certainly could not have limited the rate of glycolysis.

The techniques described in this paper provide a method for evaluating the ability of various tissues to oxidize different substrates with the production of phosphate bond energy, but the significance of the data cannot be interpreted as an assay of any single enzyme for a number of reasons. The simpler oxidative systems previously studied could be considered as having only three (12) or five (13) components, and it was relatively easy to saturate each enzyme with its proper reactant and to decide which biocatalyst was limiting the over-all rate of reaction. In the oxidative phosphorylation systems, the number of biocatalysts is much greater, and the intermediate steps are not as well understood. In the oxidative systems the *hydrogen* carriers (31) have to be in a state of dynamic balance with

an oxidized fraction and a reduced fraction, each present in sufficient amounts to saturate a specific reductant and a specific oxidant, respectively. Ordinarily this can be accomplished by adding enough of the carrier to saturate the enzyme that reacts with the smaller fraction of the carrier (12, 13). The situation is evidently analogous in the phosphorylation systems to the extent that the phosphate transfer is effected by one or more *phosphate* carriers (in the present work, ATP) which are in a state of dynamic balance between specific phosphate acceptors and donors. But the technical problem of establishing the identity of a limiting reaction in the *coupled* oxidative phosphorylation system cannot be solved in a manner analogous to that of the hydrogen transport system simply by adding a saturating amount of substrate (phosphorylated intermediate)⁶ and phosphate carrier, however, because of the presence of phosphatases which hydrolyze the phosphorylated compounds. The fact that these compounds can be acted upon by phosphatases as well as by the transphosphorylases makes the coupled systems considerably more complicated than the simple oxidative systems. The hydrolytic reactions constitute "leaks" which decrease the yield of transferable high energy phosphate ((4, 5); see also (14)). It seems likely that *in vivo* these leaks are controlled either by (1) factors affecting the activity of the phosphatases or (2) by keeping the concentration of the phosphorylated compounds at a level that is high enough to react with the specific phosphate donors and acceptors, but too low to react to a very great extent with the phosphatases. Thus from the technical standpoint we are never able to saturate *completely* the coupling systems because, as the concentration of the phosphorylated compounds is increased, the "leaks" increase also. The concentrations of ATP and oxidizable substrates employed are therefore a compromise and merely represent the levels at which the *net* phosphate uptake appears to be the best. The use of radioactive phosphorus might permit the measurement of the absolute rate of uptake.

In a system which includes many enzymes and cofactors an apparent lack of activity may be due to the absence of any one of a number of components and is therefore of little significance (*cf.* (17)). However, by adding proper amounts of as many of the known components as possible, one can increase the chances of obtaining measurable activity, and any activity obtained will constitute a minimum value for the concentration of the biocatalysts that are not added in excess.

While the activity obtained in the present work cannot be taken as an assay of any particular biocatalyst, the number of possibilities has been con-

⁶ The phosphorylated intermediates which are the donors for the transphosphorylation system are the products of oxidations which occur in the hydrogen transport system.

siderably decreased, and unless an unknown cofactor is limiting, it probably constitutes a rough measure of the transphosphorylating enzymes which couple oxidation with phosphorylation.

SUMMARY

1. Trichloroacetic acid extracts of reaction mixtures in which enzymatic oxidative phosphorylation occurred were analyzed for "true" inorganic phosphate by the direct method of Lowry and Lopez and by the indirect method of Fiske and Subbarow on fractions in which the inorganic phosphate was separated by calcium precipitation. Agreement between the two methods was excellent and it was concluded that the Lowry-Lopez method is suitable for studies on oxidative phosphorylation when identification of the compounds containing bound phosphate is not required.

2. In experiments in which the bound phosphate was largely accounted for as phosphocreatine it was shown that the bound phosphate was not significantly split off in 7 per cent trichloroacetic acid extracts at 0° over a period of several hours, which was more than ample time for the necessary manipulations.

3. The components of the reaction mixture employed for the study of oxidative phosphorylation in tissue homogenates were given in detail. Data showing the necessity of adding the fluoride last and for avoiding the presence of calcium ions were presented.

4. By employing several oxidizable substrates at once it was shown that summation of the several pathways of oxidative phosphorylation could be obtained.

5. When succinate, lactate, and glutamate were oxidized in the presence of diphosphopyridine nucleotide, homogenates of rat kidney were able to take up inorganic phosphate as rapidly as adenosine triphosphate was hydrolyzed, over a period of about 20 minutes, in the absence of fluoride. It was shown that fluoride inhibited ATP breakdown, but not ATP synthesis, under these conditions.

6. Glucose was not converted to hexose diphosphate by this system at a rate which would permit the maintenance of the ATP reservoir by the oxidation of HDP. When pure HDP was added to the system, a greatly increased rate of oxygen uptake was obtained and inorganic phosphate was esterified. The acidity resulting from glycolysis was shown to inhibit the oxygen uptake, and methods of controlling this acidity were discussed.

This work was made possible by the availability of supplies of purified adenosine triphosphate, diphosphopyridine nucleotide, and hexose diphosphate, which were all generously provided by my colleague, Dr. G. A. LePage.

Addendum—All of the work reported in this paper was done with water homogenates because it was thought that isotonic homogenates contained a large proportion of whole cells on the basis of the "cytolysis quotient" (7). It has now been found that the "cytolysis quotient" is a measure of the integrity of some subcellular entity rather than the whole cell, and that our isotonic homogenates contain very few whole cells. Isotonic KCl homogenates of liver will oxidize oxalacetic acid rapidly and will esterify inorganic phosphate concomitantly, while water homogenates will do neither. A comparison of water and isotonic homogenates with other tissues and substrates is being made.

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THE DETERMINATION OF SERUM MAGNESIUM BY THE MOLYBDIVANADATE METHOD FOR PHOSPHATE

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During the past decade there has been an increasing interest shown in the study of electrolyte balance. Magnesium, however, has played a minor rôle in these studies. There are perhaps two reasons for this; first, that serum magnesium levels remain fairly constant and, second, that magnesium determinations require special technique and equipment and are time-consuming procedures as well.

There have been three major methods used for the determination of serum magnesium, the molybdenum blue, titan yellow, and hydroxyquinoline methods.

Molybdenum Blue—Briggs (1) and Denis (2) simultaneously reported methods for the determination of serum magnesium based upon the precipitation of magnesium as magnesium ammonium phosphate, with subsequent estimation of the phosphate by the molybdenum blue method. The above procedures were employed in this laboratory with the modifications for phosphate determination used by Kuttner and Cohen (3) and Youngburg and Youngburg (4). The molybdenum blue method was abandoned chiefly because of instability of the color. A more complete discussion of this is given in a publication (5) in which the molybdivanadate method, as outlined by Kitson and Mellon (6), was used for the estimation of serum phosphate.

Titan Yellow—The titan yellow method is based on the fact that magnesium imparts a pink color to an alkaline solution of the dye (7). The clinical method of Hirschfelder and Serles (8) and the modification of Haury (9) were used, but difficulty was encountered because of the mixture of pink and yellow colors. In addition, the color was markedly affected by variations in pH and salt concentration.

Hydroxyquinoline—Hahn (10) and Berg (11) introduced the use of 8-hydroxyquinoline for the determination of magnesium. The method was further modified by the bromination technique of Bomskov (12), Greenberg and Mackey (13), and Velluz (14). Hoffman (15) discarded the bromination method in favor of a colorimetric procedure in which hydroxyquinoline combines with ferric ions in weak hydrochloric acid

solution to form a blue-green color. Magnesium hydroxyquinolate consists of such light weight material that it is extremely difficult to handle either by filtration or centrifugation and special care is necessary to avoid loss. This was the main reason for discarding the method.

In the method devised in this laboratory, a serum magnesium determination (after removal of the calcium) can be completed in $3\frac{1}{2}$ hours. Only ordinary laboratory equipment is required. The method is based on the following principle: magnesium is precipitated as magnesium ammonium phosphate and the phosphate component is estimated by the yellow color produced upon addition of vanadate and molybdate.

Procedure

Reagents—

1. Redistilled water (from an all-glass still) or distilled water known to be phosphate-free. This water was used for the preparation of all reagents.

2. Ammonium oxalate, 4 per cent aqueous solution.

3. Potassium dihydrogen phosphate, 2 per cent aqueous solution.

4. Concentrated ammonium hydroxide, redistilled.

5. Nitric acid (1:2), 1 volume of concentrated (redistilled) nitric acid plus 2 volumes of water.

6. Ammonium molybdate, 5 per cent aqueous solution. Do not heat above 50° .

7. Acid ammonium vanadate, 0.25 per cent solution in 1:2 nitric acid. Dissolve 1.25 gm. of ammonium vanadate in approximately 200 ml. of 1:2 nitric acid and heat until solution is complete. Allow the mixture to cool to room temperature and make up to a volume of 500 ml with 1:2 nitric acid.

8. Magnesium wash reagent. To 200 ml. of 95 per cent ethyl alcohol, add 50 ml. of redistilled ammonium hydroxide and make up to a volume of 1000 ml. with water.

9. Phosphate standard. Dissolve 0.560 gm. of reagent grade potassium dihydrogen phosphate (previously dried to constant weight) in redistilled water and make up to a volume of 1 liter. 1 ml. of this solution contains an amount of phosphorus equivalent to 100 γ of magnesium. Suitable dilute standards (aqueous) are prepared from the stock solution to give a range of 10 to 80 γ of magnesium per ml.

It was found necessary to redistil both the nitric acid and ammonium hydroxide, as invariably stock bottles of either of these reagents, even though labeled reagent grade, were contaminated with phosphate.

The 2 per cent phosphate and magnesium wash reagents were those used by Soffer *et al.* (16).

Procedure for Serum—Measure into a 15 ml. graduated centrifuge tube 2 ml. of unhemolyzed serum; add 2 ml. of water and 1 ml. of 4 per cent ammonium oxalate (17). Mix thoroughly and allow to stand for 2 hours. Centrifuge for 10 minutes at 2500 to 3500 R.P.M. Transfer the supernatant fluid to another centrifuge tube, being careful to remove all the solution possible without disturbing the precipitate. (Serum calcium may be determined on the calcium oxalate precipitate by the standard permanganate procedure.) To the calcium-free solution add 1 ml. of 2 per cent potassium dihydrogen phosphate and mix by agitation or swirling. Then add 1 ml. of concentrated ammonium hydroxide, invert, shake vigorously for 30 seconds, stopper, and allow to stand for 2 hours. At the end of this period the magnesium has been precipitated completely as magnesium ammonium phosphate, provided the shaking has been thorough. The tube is then centrifuged for 10 minutes at 3500 R.P.M., the supernatant fluid decanted, and the tube allowed to drain in the inverted position for 5 minutes. The precipitate and sides of the tube are washed with 5 ml. of magnesium wash reagent and the tube centrifuged as above. Two washings are necessary. After the final drainage the tube and contents are dried in an oven at 100° for 20 to 30 minutes to remove the alcohol.

To the dried precipitate in the centrifuge tube add 1 ml. of the acid vanadate reagent and agitate until the precipitate is completely dissolved. Then add 8 ml. of water and 1 ml. of 5 per cent ammonium molybdate. One may add the molybdate directly to the acid vanadate but, because of the noxious fumes arising, it is preferable to add the water first. Mix by inversion, allow to stand 5 minutes, transfer to a colorimeter tube, and read at a wave-length of 420 m μ . A blank, with 1 ml. of acid vanadate and 1 ml. of molybdate in a volume of 10 ml., is made simultaneously. The reading of the test solution after subtraction of the blank is referred to the standard curve and the number of micrograms of magnesium in the sample is calculated. This value multiplied by the factor 0.05 gives the mg. of magnesium per 100 ml. of serum.

Standard Curve—Suitable dilute standards were prepared from the stock solution such that the various samples contained amounts of phosphate equivalent to from 10 to 80 γ of magnesium. 1 ml. of the acid vanadate reagent and 1 ml. of the ammonium molybdate were added and the samples made to a volume of 10 ml. The optical densities were determined at a wave-length of 420 m μ in the Beckman quartz spectrophotometer, with 1.00 cm. Corex cells.

All results published in this paper were calculated from the optical densities observed in the Beckman spectrophotometer. Each magnesium determination was made in duplicate.

Results

Phosphate Standards—The optical densities of the standard curve, with the acid vanadate reagent, are given in Table I. In addition, a comparison is given of the optical densities of both KH_2PO_4 and MgNH_4PO_4 when the aqueous vanadate solution described in the phosphate method (5) was used.

Three observations are to be made from Table I. (1) All three solutions follow Beer's law over a range of 5 to 80 γ of magnesium. (2) The optical densities of both KH_2PO_4 and MgNH_4PO_4 solutions, calculated to contain equivalent amounts of magnesium, are identical. (3) The optical densities

TABLE I

Optical Densities (Corrected for Blank) of KH_2PO_4 and MgNH_4PO_4 with Acid and Aqueous Vanadate Solutions

The readings were made in a Beckman spectrophotometer at 420 $m\mu$.

Magnesium*	KH_2PO_4 + acid vanadate	KH_2PO_4 + aqueous vanadate	MgNH_4PO_4 + aqueous vanadate
γ			
5	0.037	0.036	
10	0.073	0.072	0.071
20	0.144	0.140	0.143
30	0.214	0.211	0.215
40	0.286	0.281	0.286
50	0.354	0.352	0.355
60	0.421	0.424	0.423
70	0.498	0.494	0.501
80	0.566	0.565	0.568
Blank	0.032	0.024	0.025

* Either the amount of magnesium equivalent to the phosphate used in the phosphate standards, or the amount of magnesium calculated to be present in the MgNH_4PO_4 .

of the standard curve (KH_2PO_4), after correction for the blank, are identical whether one uses the acid vanadate reagent or the aqueous vanadate solution.

Since a comparison of the optical densities of phosphate solutions over an 8 month period, with both the acid and aqueous vanadate solution, gave identical results, the use of the aqueous reagent has been abandoned. The only significant difference in the use of the two reagents is that the value of the blank is slightly higher for the acid vanadate.

The spectrophotometric data have previously been given (5), particularly in Figs. 2 and 3 of that paper, and need not be repeated here.

If the standard curve is prepared from readings taken on a Klett-Summer-

son photoelectric colorimeter or a Coleman junior spectrophotometer, the curve is not strictly linear. This is attributable to characteristics of these two instruments, namely, the filter and photocell. For clinical purposes these colorimeters are suitable provided proper precautions are observed in setting up the standard curve.

Color Stability—In the paper on phosphate determination (5) it was stated that the color of the blanks and test solutions was reproducible and stable for periods of at least 24 hours. This study has now been extended over a period of 4 days. During this time interval there is a slow increase in the optical density of both the blank and the test solutions. At the end of 4 days, however, the largest error due to this increase in color is only 1.5 per cent and well within the limits of error claimed for the method. Moreover, the increase in the value of the blank is not always concomitant with the increase observed in the test solution. The authors feel that this error is of minor significance, since colorimetric readings are usually made within a period of 1 or 2 hours after color development has been attained.

Effect of Time on Precipitation of Magnesium—In the precipitation of magnesium as magnesium ammonium phosphate it has long been the custom to allow precipitation to take place overnight, a period of approximately 16 hours. Preliminary studies with solutions of magnesium alone showed very little difference in recoveries during 2, 4, or 16 hour periods of precipitation when the technique already described was used. In order to prove that a 2 hour precipitation period was sufficient and could also be applied to the determination of magnesium in serum, known amounts of magnesium were added to serum and the precipitation of magnesium carried out at 2 and 16 hour intervals. Four series of determinations were made in which magnesium as magnesium chloride, in amounts ranging from 10 to 80 γ , were added to 2 ml. of serum. Calcium was removed and the magnesium determined in duplicate in each sample. The temperature at which precipitation was carried out ranged from 20–25°.

The average values for the four series are given in Table II. The results show that there is no significant difference in the 2 and 16 hour periods; the average deviation from the calculated value was ± 0.6 per cent for the 2 hour and ± 0.5 per cent for the 16 hour period. The maximum deviation of any one series ranged from -1.3 to $+2.5$ per cent.

In addition, precipitation of magnesium was carried out for 24 and 48 hour periods. The results were similar to those above if the temperature range was also similar.

Table III shows the results on a dozen sera in which magnesium was precipitated at 2 and 16 hour intervals. The 16 hour precipitation gave

lower values in five cases, higher in two, and identical results in the other five cases. The differences are typical of a larger series and also show that there is no significant difference when the 2 hour precipitation is employed for the determination of magnesium in serum.

TABLE II

Recovery of Magnesium Added to Serum; Comparison of 2 and 16 Hour Precipitations

Mg added	2 hr. precipitation, 2 ml. serum			16 hr. precipitation, 2 ml. serum		
	Mg found	Mg calculated	Recovery	Mg found	Mg calculated	Recovery
γ	γ	γ	per cent	γ	γ	per cent
0	46.0			44.1		
10	55.5	56.0	99.1	54.7	54.1	101.1
20	66.4	66.0	100.6	64.5	64.1	100.6
30	75.2	76.0	98.9	74.1	74.1	100.0
40	85.9	86.0	99.9	84.1	84.1	100.0
50	96.5	96.0	100.5	93.7	94.1	99.6
60	105.3	106.0	99.3	103.1	104.1	99.4
70	116.9	116.0	100.8	113.5	114.1	99.5
80	126.5	126.0	100.4	123.0	124.1	99.1
Average error			± 0.6			
						± 0.5

TABLE III

Comparison of 2 and 16 Hour Precipitations of Magnesium in Serum

Patient No.	2 hrs	16 hrs
	mg per cent	mg per cent
1	1.8	1.7
2	2.1	2.1
3	2.0	1.9
4	2.1	2.0
5	2.1	2.0
6	2.2	2.1
7	2.1	2.1
8	1.9	2.0
9	2.0	2.0
10	2.1	2.1
11	2.1	2.2
12	1.8	1.8

Neubauer (18) showed that when magnesium is precipitated as magnesium ammonium phosphate in the cold the results are sometimes high, while at other times the results are low. A series of 2 and 16 hour precipitations of magnesium added to serum was carried out at 4°, 25°, and

37°. The results at 25° and 37° were identical for both time intervals. At 4° the magnesium content ranged from 3 to 4 per cent higher, showing that the lower temperature does affect the nature of the precipitate formed. The magnesium ammonium phosphate formed at ordinary laboratory temperatures was probably slightly contaminated with the tribasic phosphate, $\text{Mg}_3(\text{PO}_4)_2$, which may have formed because of the lower temper-

TABLE IV
Serum Magnesium Values of Normal Adults

Patient No	Mg per 100 ml serum	
	Males	Females
	mg.	mg.
1	2.0	1.8
2	2.0	2.1
3	2.0	2.1
4	2.1	2.0
5	2.1	2.2
6	2.2	1.8
7	1.9	1.9
8	2.3	2.2
9	2.1	2.1
10	1.9	2.0
11	1.8	1.8
12	2.2	2.3
13	2.0	2.0
14	2.2	2.0
15	2.3	1.8
16	2.0	1.8
17	2.0	2.1
18	2.2	1.9
19	2.0	1.7
20	2.0	2.1
21	1.9	1.9
Average	2.06	1.98

ature used. This would readily account for the higher values obtained when the precipitation was carried out at 4°.

Recovery of Magnesium Added to Serum—The results in Table II show the excellent recovery of magnesium added to serum as well as a comparison of the 2 and 16 hour periods of precipitation. In addition to magnesium chloride, recovery studies were made in which magnesium ammonium phosphate was added in amounts ranging from 10 to 80 γ of magnesium. With the latter compound the recoveries all showed a positive deviation but the maximum error in any one of the three series was +2.5 per cent.

Serum Magnesium of Normal Adults—Blood samples were obtained from approximately the same members of the laboratory staff as in the phosphate determinations (5). There were forty-two adults in the group, ranging in age from 20 to 60 years. There was equal distribution between the sexes. The values obtained ranged from 1.7 to 2.3 mg. of magnesium per 100 ml. of serum. The results are given in Table IV.

DISCUSSION

The results obtained by this new method show a much narrower range than those reported by the majority of other workers. Average values or normal ranges (mg. per cent) in close agreement with those reported in this paper are given by Briggs (1), 2.23 to 2.50; Denis (2), 2.0; Weissenbach *et al.* (19), 1.8 to 2.2; Roy (20), 2.48; and Kramer and Tisdall (17), 1.80 to 2.30. Of the authors using the hydroxyquinoline method, those whose results are in closest agreement with values by the vanadate method are Hoffman (15), 1.90 to 2.50; Bomskov (21), 1.7 to 2.6; and Velluz and Velluz (22), 1.60 to 2.40. Greenberg and his associates (23) report a higher range, 2.0 to 3.6. With the exception of the values reported by Hirschfelder and Haury (24), 1.80 to 2.40, results by the titan yellow method show the greatest discrepancy from the values reported in this paper as judged from the results of Haury (25), 1.74 to 3.10, and Bernstein and Simkins (26), 1.23 to 3.54.

Haury (27) has given a thorough review of the variations in serum magnesium in health and disease. Because of the wide variations reported by the different methods used for its determination, many of the studies reported should be reevaluated.

It is our belief that normal magnesium levels cover a very narrow range and remain fairly constant. In spite of many reports of the variations of magnesium in different diseases, little is known about its exact physiological function. Further studies are necessary and should prove to be of significant value.

The advantages of the method, herein reported, are as follows: (1) after removal of the calcium, a serum magnesium determination may be completed in approximately $3\frac{1}{2}$ hours, (2) the technique is simple and no special equipment is needed, (3) the color is stable over a long period of time and duplicate determinations show excellent agreement, and (4) the error of the method as determined by the recovery studies gives an average deviation of ± 0.55 per cent from the calculated value.

SUMMARY

1. A method is described in which magnesium is precipitated from serum as magnesium ammonium phosphate with subsequent determination

of the phosphate component by the molybdivanadate procedure. After removal of the calcium, a magnesium determination may be completed in $3\frac{1}{2}$ hours. Only ordinary laboratory equipment is necessary.

2. The solutions obey Beer's law and the method may be used for concentrations of magnesium ranging from 10 to 80 γ .

3. The maximum error of the method ranges from -1.3 to $+2.5$ per cent, with an average deviation of ± 0.55 per cent from the calculated value.

4. The serum magnesium in forty-two normal adult males and females (sex distribution being equal) ranged from 1.7 to 2.3 mg. per 100 ml. The values for females were only slightly lower than those for males.

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THE METABOLIC INTERRELATIONS OF THYROXINE AND DIIODOTYROSINE IN THE THYROID GLAND AS SHOWN BY A STUDY OF THEIR SPECIFIC ACTIVITY-TIME RELATIONS IN RATS INJECTED WITH RADIOACTIVE IODINE*

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The evidence in support of the hypothesis that thyroxine is formed in the thyroid from tyrosine with diiodotyrosine as an intermediate has recently been reviewed by Harington (1). The presence of relatively large amounts of diiodotyrosine in the thyroid gland and, in particular, the finding that natural thyroxine and natural tyrosine are configuratively related are considered by him to favor strongly such a mechanism. Further support is offered by the isolation of thyroxine from iodinated proteins (2-4) and by the finding that thyroxine is formed during the prolonged incubation of diiodotyrosine in a slightly alkaline solution (5-10).

Zilversmit *et al.* have devised a procedure based on the use of labeling agents for testing whether one compound in a tissue is a precursor of another (11). This involves a study of changes in the specific activities of the two compounds with time. With the aid of methods recently developed in this laboratory for fractionating the iodine in as little as 20 mg. of thyroid tissue (12) and with the aid of radioactive iodine, it became possible to apply this procedure to a study of the interrelations of diiodotyrosine and thyroxine in the thyroid glands of the rat.

EXPERIMENTAL

Rats of the Slonaker and Curtis-Dunning strains weighing 180 to 250 gm. were used in this study. All had been raised on the same diet. Each rat received by way of the tail vein 0.5 to 1.0 cc. of an isotonic solution containing 20 microcuries of I^{131} per cc. with no I^{127} carrier. The rats were sacrificed in groups of three or four at the following intervals thereafter: 15 minutes, 1 hour, 4, 14, and 50 hours.

Fractionation of Iodine—The rats were anesthetized with nembutal, bled by severing the heart and great vessels, and their thyroids removed. The glands were then rapidly weighed and homogenized with 1 cc. of cold 10 per cent trichloroacetic acid in a small glass homogenizer. The homogenate together with the water washings used in its preparation

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was transferred to a graduated centrifuge tube. The protein precipitate was separated by centrifugation and washed once with 5 cc. of 5 per cent trichloroacetic acid. The supernatants were combined for the determination of *non-protein-bound iodine*. The precipitate was hydrolyzed with 1 cc. of 2 N NaOH on the steam bath for 10 hours and separated into diiodotyrosine and thyroxine fractions in the manner previously described

TABLE I
Distribution of I^{131} and I^{127} in Rat Thyroids

Interval after injection of I^{131}	Radioactive iodine (I^{131})							Chemically measured iodine (I^{127})					
	Per cent of administered I^{131} recovered in thyroids				Per cent of total thyroid I^{131} in			Amount of I^{127} in			Per cent of total thyroid I^{127} in		
	Total	Thyroxine fraction	Diiodotyrosine fraction	Non-protein-bound fraction	Thyroxine fraction	Diiodotyrosine fraction	Non-protein-bound fraction	Thyroxine fraction	Diiodotyrosine fraction	Non-protein-bound fraction	Thyroxine fraction	Diiodotyrosine fraction	Non-protein-bound fraction
hrs.								γ	γ	γ			
0.25	1.55	0.22	1.26	0.07	14.5	81.1	4.4	2.9	7.5	0.43	27	69	4
0.25	1.64	0.21	1.35	0.08	12.9	82.0	5.1	4.1	10.4	0.51	27	69	4
0.25	1.54	0.16	1.31	0.08	10.0	85.0	5.0	3.3	8.3	0.49	27	68	5
1	4.22	0.66	3.45	0.10	15.6	82.1	2.3	2.5	7.6	0.40	24	72	4
1	4.47	0.82	3.53	0.12	18.3	79.0	2.7	2.8	7.8	0.53	25	70	5
1	3.37	0.60	2.68	0.09	17.9	79.3	2.8	2.8	7.4	0.35	27	70	3
1	3.61	0.66	2.84	0.12	18.2	78.7	3.1	1.5	11.4	0.57	27	69	4
4	9.60	2.1	7.33	0.21	21.7	76.1	2.2	3.5	8.3	0.41	29	68	3
4	7.43	1.4	5.91	0.13	18.5	79.7	1.8	2.4	6.8	0.40	25	71	4
4	8.10	1.4	6.57	0.14	17.1	81.1	1.8	2.7	7.7	0.43	25	71	4
14	16.3	4.3	11.7	0.29	26.1	72.1	1.8	4.7	10.9	0.67	29	67	4
14	19.5	4.9	14.4	0.31	21.8	73.7	1.5	4.2	10.0	0.78	28	67	5
14	19.2	4.5	14.3	0.38	23.3	74.7	2.0	5.0	12.0	0.86	28	67	5
14	21.4	5.7	15.3	0.39	26.4	71.7	1.9	3.2	8.6	0.78	25	68	7
50	11.9	3.3	8.35	0.22	28.0	70.1	1.9	3.2	8.8	0.71	25	69	6
50	12.9	3.5	9.20	0.16	27.4	71.3	1.3	2.7	6.5	0.32	28	68	4
50	10.5	2.8	7.55	0.15	26.4	72.1	1.4	2.5	8.1	0.31	23	74	3
50	13.5	3.6	9.70	0.22	26.3	72.0	1.7	2.5	7.7	0.39	24	73	3

(12). The amounts of iodine and the radioactivity in each fraction were then determined.

Results

Uptake of Radioactive Iodine—The percentages of the administered I^{131} recovered in the different iodine fractions of the gland are shown in Table I. In 15 minutes the thyroid glands already contained 1.5 per cent of the injected dose. The maximum uptake of total iodine (about 20 per cent of the injected dose) occurred between 14 and 50 hours.

Distribution of Chemical Iodine (I^{127})—The percentages of the gland's

iodine present in the various fractions are shown in Table I. The distribution of the iodine between the thyroxine and diiodotyrosine fractions was found to be strikingly constant in the different animals. The thyroxine iodine varied only from 23 to 29 per cent of the total; the mean was 26 and its standard error 0.43. Diiodotyrosine iodine varied from 67 to 74 per cent, with a mean and standard error of 69 ± 0.48 . These findings agree well with the results of a previous report from this laboratory in which it was demonstrated that not only is there a constant percentage of the total iodine present in the thyroxine fraction but this percentage remains constant even when the total gland iodine is augmented by increasing the iodine intake (13).

Distribution of Radioactive Iodine (I^{131})—There was also a fairly constant distribution of radioactive iodine between the thyroxine and diiodotyrosine fractions of the thyroid at each interval. The exceedingly rapid rate of appearance of the injected I^{131} in the organic iodine fraction, however, was unexpected. As early as 15 minutes after the injection, 95 per cent of the radioactivity present in the thyroid was precipitable with trichloroacetic acid, and therefore presumed to be organically bound. At this early interval the greatest part of the activity, namely over 80 per cent, was already in the diiodotyrosine fraction, whereas 10 to 15 per cent was in the thyroxine fraction. At all intervals up to 50 hours the fraction of radioiodine precipitable as trichloroacetic acid constituted over 95 per cent of the thyroid's total activity. The relative amount of activity in the thyroxine fraction increased constantly, and in 50 hours this fraction contained over 25 per cent of the gland's total radioactivity.

Criteria for Establishing Identity of Precursor As Applied to Diiodotyrosine Iodine and Thyroxine Iodine after Injection of Single Dose of Radioactive Iodine

The graphical procedure of Zilversmit *et al.* for testing the identity of a precursor is based upon the analysis of the specific activity-time curves of the suspected precursor and product (11). Such curves are best drawn when all the measurements are carried out on a single animal. Since this was not feasible in the present experiment, several rats were sacrificed at each interval and the average of the individual specific activities used for plotting the curves in Fig. 1. The individual values calculated from the data in Table I are shown in Table II. Good agreement between the individual values in each group made it possible to draw satisfactory curves depicting the changes in specific activity with time.

Zilversmit *et al.* have demonstrated that in a case in which Compound A is the immediate precursor of Compound B the following three conditions must hold in an experiment in which a *single dose of a labeling agent is administered*: (1) The specific activity of Compound A is greater

TABLE II
Specific Activity Data

Interval after iodine injection hrs.	Thyroxine fraction		Diiodotyrosine fraction	
	counts per sec	per γ iodine	counts per sec	per γ iodine
0.25	38		82	
0.25	25		64	
0.25	23		77	
1	129		222	
1	143		222	
1	105		177	
1	72		122	
4	292		432	
4	281		426	
4	252		419	
14	413		527	
14	567		705	
14	438		585	
14	865		873	
50	510		465	
50	640		692	
50	545		456	
50	696		619	

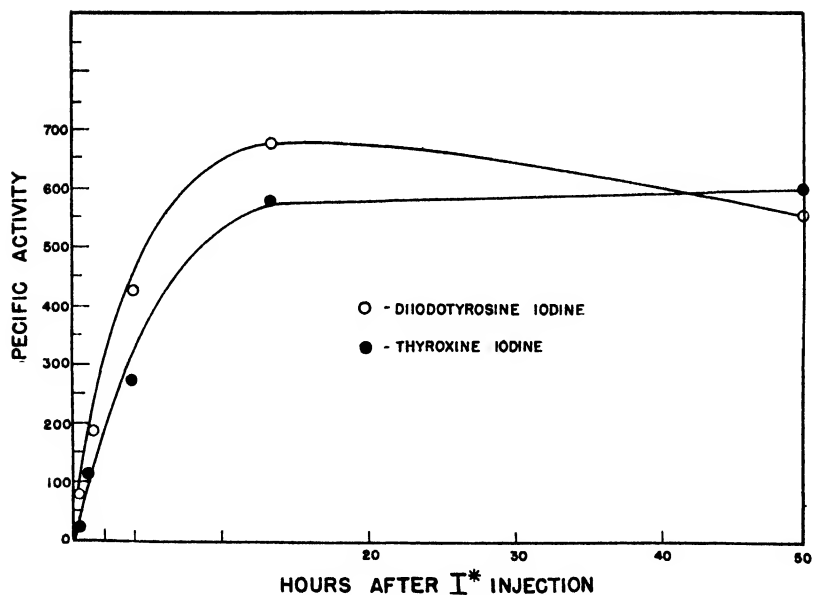


FIG. 1 Specific activity-time relations for diiodotyrosine iodine and thyroxine iodine

than that of Compound B before the latter reaches its maximum specific activity. (2) The specific activity of Compound A equals that of Compound B when the specific activity of Compound B has reached its maximum specific activity. (3) After the specific activity of Compound B has reached its maximum, the specific activity of Compound B is greater than that of Compound A.

Fig. 1 shows that within the limits of experimental error these three conditions are fulfilled for diiodotyrosine as Compound A and for thyroxine as Compound B. The data presented here therefore provide support for the view that diiodotyrosine is the biological precursor of thyroxine.

DISCUSSION

Mann, Leblond, and Warren were the first to use radioactive iodine to determine whether diiodotyrosine is the precursor of thyroxine (14). These workers injected six dogs with tracer doses of radioactive iodine and sacrificed two at each of the following intervals: 0.5, 8, and 48 hours. The thyroid iodine was separated into three fractions, inorganic, thyroxine, and diiodotyrosine iodine, and the specific activities of the iodine in each fraction determined. Because the uptake of I^{131} by the thyroids of these dogs varied tremendously, it was not feasible to compare directly the specific activities among the different animals. To permit a comparison to be made, Mann *et al.* introduced a new ratio which they designated "relative specific activity." The latter is the ratio of the specific activity of the iodine of a given fraction to the specific activity of total iodine of the gland.¹ The relative specific activities of the various iodine fractions were then plotted against time and the resulting curves interpreted as if they represented ordinary specific activity-time curves. As evidence in support of a precursor-product relation between diiodotyrosine and thyroxine, Mann *et al.* then cite their finding of a linear increase in the relative specific activity of thyroxine iodine coincident with an approximate constancy in the relative specific activity of diiodotyrosine iodine. The validity of this evidence, however, appears to us to be open to question. When relative specific activities were calculated from the data of Table I, a linear increase in the values for thyroxine iodine with time was not observed, even though those for diiodotyrosine iodine remained approximately constant. In attempting to resolve this discrepancy the following should be noted²:

¹ It is necessary to call attention here to the fact that this meaning of the term "relative specific activity" differs from that assigned to it by Hevesy (15), who first introduced this term.

² We are indebted to D. B. Zilversmit for valuable assistance in the evaluation of the "relative specific activity" data of Mann *et al.* (14).

1. The straight line obtained by Mann *et al.*, when relative specific activities of thyroxine iodine were plotted against time, was based on only three points and did not pass through the origin.

2. When the specific activity of the precursor of a given compound is kept constant, the specific activity of this compound increases linearly only so long as an appreciable amount of the compound does not leave the tissue. Since the experiments of Mann *et al.* lasted for 48 hours, it is unlikely that in this interval only a negligible amount of labeled thyroxine left the gland. We have shown elsewhere (16) that in the rat labeled iodide removed from plasma by the thyroid gland reappears rapidly in plasma as protein-bound iodine (presumably thyroxine).

3. The constancy observed in the values for the relative specific activity of diiodotyrosine iodine is a coincidence. Since the largest part of the iodine in the gland, both in radioactive and stable form, is present as diiodotyrosine, this constancy is inherent in the method of calculation.

4. Thyroxine iodine amounted to 6.5 and 10 per cent of the total iodine of the thyroid gland in the two dogs sacrificed at the 48 hour interval, but in the two sacrificed at the 8 hour interval it constituted 26 and 33 per cent. The relative specific activities of thyroxine iodine and diiodotyrosine iodine agreed fairly well for the two dogs sacrificed at each interval, but it is questionable whether agreement would have been obtained *if all four dogs had been sacrificed at the same interval*. Agreement under the latter conditions, *i.e.* in a large number of dogs with widely varying percentages of thyroxine in their thyroids and sacrificed at the same interval after the injection of I^{131} , must be demonstrated to justify the use of relative specific activities.

5. It is curious that despite a 10-fold variation in the thyroxine content (65.6 to 643 γ of iodine) of the thyroids of their dogs, Mann *et al.* calculated from the relative specific activity-time curves that a constant *percentage* (1.55 per cent) of the thyroxine of the glands was renewed every hour. This implies a 10-fold variation in thyroxine turnover rate, although the body weight of these dogs varied only 2-fold.

In the present study a direct comparison of specific activities of iodine in the diiodotyrosine and thyroxine fractions of the gland was made possible by the use of a uniform group of carefully controlled rats. Since at each interval (see Table II) the three to four values obtained for the specific activities of diiodotyrosine or for thyroxine were in fairly good agreement, it was permissible to plot their averages against time, as shown in Fig. 1.

It was shown by Zilversmit *et al.* (11) that curves of the type shown in Fig. 1 can also be used to calculate the time required for the complete renewal of the thyroxine in the gland (turnover time). The assumptions

underlying this calculation have been fully discussed elsewhere (11). When this calculation was carried out in the present experiment, the turnover time of thyroxine amounted to approximately 24 hours. If we assume that only a small fraction of the thyroxine is destroyed within the thyroid gland, then this result means that in 24 hours the thyroid gland secretes an amount of thyroxine equal to that present in the gland. Since the average thyroxine iodine content of the thyroid gland was 3.3 γ , the rate of thyroxine iodine secreted, as calculated by this method, is approximately 1.5 γ per 100 gm. of body weight per 24 hours. This value is lower than that found by Dempsey and Astwood (5.2 γ of *l*-thyroxine per 100 gm. of body weight per 24 hours (17)) but higher than that reported by Griesbach and Purves (2.25 γ of *dl*-thyroxine per 100 gm. of body weight per 24 hours (18)). The methods used by these workers (17, 18) were based on a different principle from the one described here.

The fraction termed here non-protein-bound iodine is probably not a discrete thyroid fraction but a mixture of inorganic iodine and some organic iodine that fails to precipitate with the proteins. This fraction constituted only about 5 per cent of the total iodine (I^{127}) and about the same percentage of the total radioiodine (I^{131}). Since inorganic I^{131} added to the thyroid before homogenization was quantitatively recovered in the trichloroacetic acid supernatant, it may be assumed that all the inorganic iodide of the gland appears in this fraction. But not all of the iodine in this fraction behaved like inorganic iodide. When trichloroacetic acid extracts were prepared from the glands of rats that had been injected with I^{131} , only 20 to 30 per cent of the I^{131} in the extracts was oxidized to I_2 by the addition of iodide carrier and excess iodate. This means that only about one-fourth of the radioiodine in this fraction can be considered to be in the form of iodide.

For the purpose of the specific-activity determinations carried out here, however, the treatment of the glands with trichloroacetic acid introduced no error. This procedure served to separate the inorganic iodine from the protein-bound iodine of the precipitate, and the small loss of organic iodine from the precipitate would not be expected to affect the specific activities of the thyroxine and diiodotyrosine iodine.

We are grateful to Dr. Hardin B. Jones for assistance in carrying out the experiments described here.

SUMMARY

1. Specific activity-time curves were constructed for the iodine of the thyroxine and diiodotyrosine fractions of the thyroid glands of rats that had received a single intravenous injection of radioactive iodine. The

curves obtained satisfied certain previously established criteria for a precursor-product relationship, thus providing further experimental evidence for the view that diiodotyrosine is the biological precursor of thyroxine.

2. The average rate of thyroxine secreted by the thyroid gland of the rat as calculated from specific activity-time curves was found to be about 2 γ per 100 gm. of body weight per 24 hours.

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BIOCHEMICAL TRANSFORMATIONS AS DETERMINED BY COMPETITIVE ANALOGUE-METABOLITE GROWTH INHIBITIONS

VI. PREVENTION OF BIOTIN SYNTHESIS BY 2-OXO-4- IMIDAZOLIDINECAPROIC ACID

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Much attention has been given to the possibility that desthiobiotin is a precursor of biotin in biological systems, since Melville, Dittmer, Brown, and du Vigneaud (1) first reported that desthiobiotin was equally as effective as biotin in stimulating the growth of *Saccharomyces cerevisiae*. Dittmer, Melville, and du Vigneaud (2) later demonstrated that desthiobiotin was converted by yeast to some substance, presumably biotin, which could replace the biotin requirement of *Lactobacillus casei*, an organism which cannot utilize desthiobiotin. Evidence of a similar nature was presented by Leonian and Lilly (3). Stokes and Guinness (4) reported that desthiobiotin could substitute for biotin in the nutrition of a fungus and of a number of yeasts, but that it did not support growth of the three bacteria tested by them. The most convincing evidence that desthiobiotin is a normal intermediate in the synthesis of biotin was presented by Tatum (5). By subjecting *Penicillium chrysogenum* to x-rays a biotin-requiring strain was obtained which could not utilize desthiobiotin. This organism, when grown on minimal quantities of biotin, produced a substance which was identical with desthiobiotin in its growth-promoting properties for several other organisms, including *Penicillium notatum*, strain 21464.

In the present investigation a study has been made of the inhibition of growth of *Escherichia coli* by 2-oxo-4-imidazolidinecaproic acid¹ and the effect of biotin and desthiobiotin on this toxicity. Dittmer and du Vigneaud (6) synthesized 2-oxo-4-imidazolidinecaproic acid and reported its feeble "antibiotin" effect for both *Lactobacillus casei* and *Saccharomyces cerevisiae*. Later, Duschinsky and Dolan (7) synthesized the compound by an improved method and reported inhibitory action in close agreement with the results of Dittmer and du Vigneaud.

Application of the theory of *inhibition analysis* (8) to the effect of des-

¹ The nomenclature of *Chemical Abstracts* is used instead of 4-(imidazolidone-2) caproic acid (6) and 5-carboxyamyl-2-imidazolidone (7).

thiobiotin and biotin on the toxicity of 2-oxo-4-imidazolidinecaproic acid for *Escherichia coli* indicates that the biosynthesis of biotin must proceed through desthiobiotin as an intermediate, and that no alternative mechanism is available to the organism. This inhibitor evidently prevents the biosynthesis of biotin by competing with desthiobiotin for the enzyme system which converts desthiobiotin to biotin.

TABLE I
Growth Inhibition by 2-Oxo-4-imidazolidinecaproic Acid and Its Reversal by Desthiobiotin

Test organism, *Escherichia coli*; incubated 15 hours at 37°.

<i>dl</i> -2-Oxo-4-imidazolidinecaproic acid	<i>dl</i> -Desthiobiotin	Galvanometer reading*
γ per 5 cc.	γ per 5 cc.	
0	0	42.5
100	0	43 0
300	0	2.5
1,000	0	2.5
0	3	40.0
100	3	42.5
300	3	25 0
1,000	3	2 0
0	10	43.0
100	10	40.0
300	10	41 5
1,000	10	2 5
0	30	43 5
300	30	41.5
1,000	30	42.0
3,000	30	2.5
0	100	40 0
1,000	100	40 0
3,000	100	40 0
10,000	100	2 0
10,000	1000	42 5

Antibacterial index = 100

* A measure of culture turbidity, distilled water reads 0, an opaque object 100.

EXPERIMENTAL

Materials

The 2-oxo-4-imidazolidinecaproic acid (300 mg.) was kindly supplied to us by Dr. Robert Duschinsky (7).

Biotin was obtained from Merck and Company, Inc., and *dl*-desthiobiotin was obtained from Hoffmann-La Roche, Inc.

Testing Methods

The basal medium used in tests with *Escherichia coli* has been previously described (8). The casein hydrolyzed with trypsin was omitted. The organism used was carried by daily transfers on the inorganic salts-glucose medium (6). In order to conserve material, these tests were carried out with cultures of 5 cc. total volume.

TABLE II

Prevention of Toxicity of 2-Oxo-4-imidazolidinecaproic Acid by Biotin

Test organism, *Escherichia coli*; incubated 15 hours at 37°.

<i>dl</i> -2-Oxo-4-imidazolidinecaproic acid	Biotin	Galvanometer reading
γ per 5 cc.	γ per 5 cc.	
0	0	42.5
100	0	43.0
300	0	2.5
300	0.003	2.5
300	0.01	11.0
300	0.03	13.0
300	0.1	13.0
0	0.03	42.5
300	0.03	40.5
1,000	0.03	24.5
3,000	0.03	15.0
10,000	0.03	6.0
0	0.1	39.0
300	0.1	42.5
1,000	0.1	42.0
3,000	0.1	41.0
10,000	0.1	38.0

Antibacterial index > 10,000

Results

As shown in Table I, 2-oxo-4-imidazolidinecaproic acid at a concentration of 300 γ per 5 cc. completely prevented growth of *Escherichia coli* in a salts-glucose medium. The toxicity was prevented competitively by desthiobiotin, the antibacterial index (9) being approximately 100 over a wide range of concentrations. Even 10 mg. of the inhibitor per 5 cc. were not toxic to the organism in the presence of sufficient (1 mg.) desthiobiotin; thus, inhibition of growth was apparent only when the ratio of inhibitor to desthiobiotin exceeded a definite value.

The effect of biotin on the toxicity of 2-oxo-4-imidazolidinecaproic acid is indicated in Table II. At least 0.03 γ of biotin per 5 cc. was necessary to

prevent the toxicity of the inhibitor at concentrations of 300 γ per 5 cc., the lowest concentration preventing growth in the absence of biotin. In the presence of 0.03 γ of biotin per 5 cc., 2-oxo-4-imidazolidinecaproic acid was toxic only at relatively high concentrations, 3 to 10 mg. per 5 cc. An increase in the concentration of biotin to 0.1 γ per 5 cc. prevented the toxic action of the inhibitor, even at 10 mg. per 5 cc. The results indicate that the minimal amount of biotin necessary to obtain any prevention of toxicity of the inhibitor was not much less than the amount which is effective at all levels. If 2-oxo-4-imidazolidinecaproic acid prevented a function of desthiobiotin other than biotin synthesis, the antibacterial index was greater than 10,000

DISCUSSION

From the results, it is apparent that 2-oxo-4-imidazolidinecaproic acid, which differs from desthiobiotin by lacking a single methyl group, competes with desthiobiotin for an enzyme system, the function of which is essential for growth. The antibacterial index for this competitive inhibition is about 100.

It has been proposed (8) that the antibacterial index is a function of the inhibited enzyme system which is the limiting process for growth, and that the addition of the product of this specific limiting enzyme system to the organism in excess of growth requirements either completely prevents the toxicity of the analogue at any concentration or results in a higher antibacterial index which is a function of another enzyme system for which the analogue and metabolite compete.

The effect of biotin on the toxicity of 2-oxo-4-imidazolidinecaproic acid apparently corresponds to that of the product of the inhibited enzyme system, and no other essential enzyme system is affected by the inhibitor. Hence, it appears that 2-oxo-4-imidazolidinecaproic acid prevents the biosynthesis of biotin in *Escherichia coli* by competing with the desthiobiotin for the enzyme system which converts desthiobiotin to biotin. It would therefore appear that desthiobiotin is the normal precursor of biotin, and since biotin synthesis limits the growth of the organism when the conversion of desthiobiotin to biotin is blocked, there appears to be no other efficient alternative method of biosynthesis of biotin in the organism.

SUMMARY

2-Oxo-4-imidazolidinecaproic acid prevents the growth of *Escherichia coli* by competing with desthiobiotin for an enzyme system, the functioning of which is essential for biotin synthesis. The antibacterial index is approximately 100 in the absence of biotin. In the presence of biotin in

slight excess of growth requirements the inhibitor is no longer toxic to the organism, even at concentrations approaching saturation. The normal biosynthesis of biotin proceeds through this enzyme system with desthiobiotin, and no efficient alternative system appears to be immediately available to the organism.

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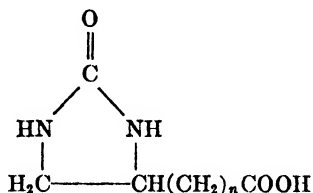
ANTIBIOTIN ACTIVITY OF IMIDAZOLIDONE ALIPHATIC ACIDS*

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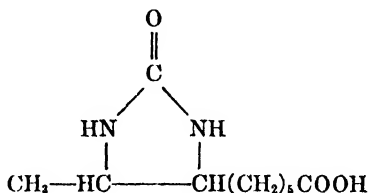
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The synthesis of four imidazolidone aliphatic acids (I) has been described in a recent communication from this laboratory (1). The relationship between the structure of these acids and desthiobiotin (II) is illustrated



(I)

Imidazolidonevaleric acid ($n = 4$)
 Imidazolidonecaproic acid ($n = 5$)
 Imidazolidoneheptanoic acid ($n = 6$)
 Imidazolidoneoctanoic acid ($n = 7$)



(II)

Desthiobiotin

by the accompanying structures. It has already been reported that *dl*-imidazolidonecaproic acid (2, 3) is an antibiotin for *Lactobacillus casei* and *Saccharomyces cerevisiae*.

It is the purpose of the present paper to report the relative antibiotin activities of these four imidazolidone aliphatic acids against *Lactobacillus casei* and *Saccharomyces cerevisiae* and to describe in detail the microbiological methods used for the determination of these values. In addition, the avidin-combining abilities of these compounds will be discussed.

EXPERIMENTAL

Antibiotin Activity of Imidazolidone Aliphatic Acids—The antibiotin activity of a compound is expressed in this paper as the molar inhibition ratio (2), which is defined as the number of molecules of an antibiotin required to inhibit 1 molecule of biotin. The antibiotin activity is deter-

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mined experimentally by finding the amount of an antibiotin which is able to reduce the growth obtained with 0.0002 γ of biotin to a level equivalent to that obtained with 0.0001 γ of biotin. The molar inhibition ratio can then be calculated after the conversion of these amounts to molar quantities. The smaller the inhibition ratio, the greater the antibiotin activity of a given compound.

The inhibition of the growth of yeast by three of the imidazolidone aliphatic acids is illustrated by the curves of Fig. 1. All tests were made

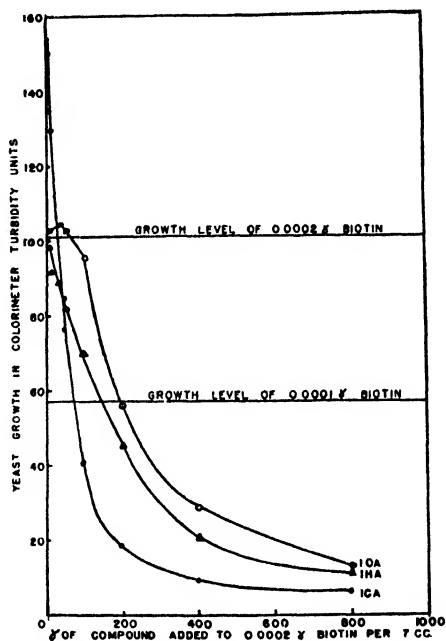


FIG. 1. Relative antibiotin activity of imidazolidonecaproic acid (Curve ICA), imidazolidoneheptanoic acid (Curve IIA), and imidazolidoneoctanoic acid (Curve IOA) on the growth of *Saccharomyces cerevisiae*.

with *Saccharomyces cerevisiae*, Fleischmann strain 139, with the basal medium described by Snell, Eakin, and Williams (4), with the following additions per liter of medium: L-aspartic acid 100 mg., casein hydrolysate (Smaco) 5.0 cc., L-tryptophan 10 mg., sodium acetate 600 mg., thiamine chloride 20 γ , pyridoxine hydrochloride 20 γ , calcium pantothenate 20 γ , and nicotinic acid 40 γ . For convenience, this medium will be designated as "C-14". The inoculum was 0.6 mg. of moist yeast (from a 24 hour culture grown on wort agar) per 100 cc. of medium. The incubation period was 16 hours at 30°. The tests were carried out in 20 X 150 mm.

Pyrex test-tubes, each of which contained 1 cc. of addenda and 6 cc. of medium; the tubes were incubated in a slanted position to permit better aeration.

The effect of increasing amounts of biotin in nullifying the inhibitory action of the imidazolidone aliphatic acids on the growth of yeast is illustrated by the curves of Fig. 2.

A study of the inhibitory action of the imidazolidone aliphatic acids on the growth of *Lactobacillus casei* was made with the medium described by Shull, Hutchings, and Peterson (5, 6). The inoculum was grown in the medium described by these authors; a 24 hour culture was centrifuged, washed, and resuspended in 10 cc. of sterile saline; 1 drop of a 1:20 dilution

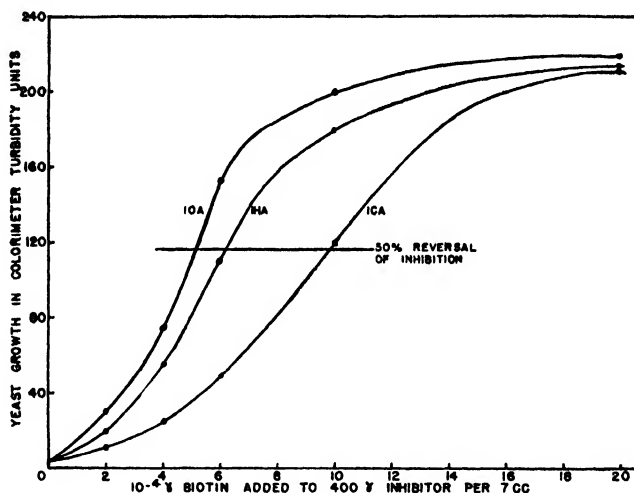


FIG. 2. Nullification of the antibiotic activity of the imidazolidone aliphatic acids by biotin. Curve IOA, imidazolidoneoctanoic acid, Curve IIIA, imidazolidoneheptanoic acid, and Curve ICA, imidazolidonecaproic acid

of this suspension was used per tube. During most of the antibiotic studies with *L. casei*, the incubation period was 40 to 44 hours. When the test cultures were permitted to grow for 72 hours at 37° , the inhibition ratio was somewhat higher, but the relationship between the compounds remained the same. Just as in the studies with yeast, the addition of larger amounts of biotin prevented the inhibitory action on *L. casei* of all these compounds. The relative antibiotic activities of the *dl*-imidazolidone aliphatic acids for *L. casei* and *Saccharomyces cerevisiae* are tabulated in Table I.

That desthiobiotin is equally as effective as biotin in stimulating the growth of *Saccharomyces cerevisiae* has already been reported (7) and

confirmed (8, 9). The imidazolidone aliphatic acids were much more effective in inhibiting the yeast growth when desthiobiotin was the stimulant than when an equal amount of biotin was used. The molar inhibition ratios of these compounds to desthiobiotin were as follows: imidazolidone-

TABLE I
Relative Antibiotic Activities of Imidazolidone Aliphatic Acids

Compound	Molar inhibition ratio*	
	<i>S. cerevisiae</i>	<i>L. casei</i>
<i>dl</i> -Imidazolidonecarboxylic acid	Inactive	Inactive
<i>dl</i> -Imidazolidonevaleric acid	>37,000,000	52,000,000
<i>dl</i> -Imidazolidonecaproic "	850,000	100,000
<i>dl</i> -Imidazolidoneheptanoic acid	1,700,000	1,600,000
<i>dl</i> -Imidazolidoneoctanoic "	2,000,000	10,000,000

* These molar inhibition ratios represent average values from several experiments, they are reproducible within 20 per cent.

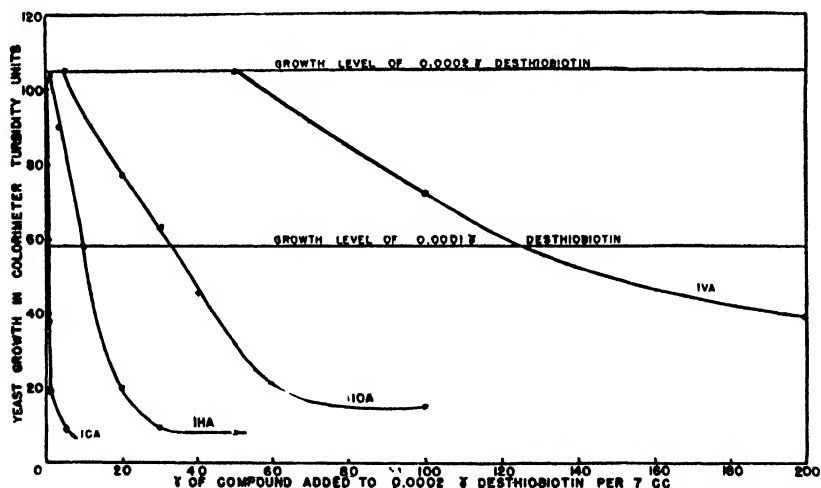


FIG. 3. Yeast growth inhibition curves showing the antagonism between imidazolidone aliphatic acids and desthiobiotin. Curve ICA, imidazolidonecaproic acid, Curve IHA, imidazolidoneheptanoic acid, Curve IOA, imidazolidoneoctanoic acid, and Curve IVA, imidazolidonevaleric acid.

valeric acid 1,340,000, imidazolidonecaproic acid 3000, imidazolidoneheptanoic acid 100,000, and imidazolidoneoctanoic acid 270,000. The inhibitory effects of these compounds on the growth of yeast when desthiobiotin was the stimulant are illustrated by the curves of Fig. 3. When the data of Fig. 3 are compared with those of Fig. 1, it will be noted that

the imidazolidone aliphatic acids are many times more effective against desthiobiotin than against biotin for the growth of *Saccharomyces cerevisiae*. Again, imidazolidonecaproic acid is the most active.

The antagonism between the imidazolidone aliphatic acids and desthiobiotin with resultant inhibition of yeast growth can be prevented by the addition of more desthiobiotin. 0.034 γ of desthiobiotin was required to counteract half-way the toxicity of 5 γ of imidazolidonecaproic acid; 0.0012 and 0.00045 γ respectively of desthiobiotin were required to counteract half-way the toxicity of 50 γ of imidazolidoneheptanoic and imidazolidoneoctanoic acids, whereas only 0.001 γ of desthiobiotin was necessary to counteract half-way the toxicity of 500 γ of imidazolidonevaleric acid.

Imidazolidonecaproic acid was also tested for its antibiotin effect on types I, II, and III pneumococci. The pneumococci were grown in the

TABLE II
Inhibition of Growth of Pneumococci by Imidazolidonecaproic Acid

Imidazolidonecaproic acid mg	Growth of pneumococci*		
	Type I	Type II	Type III
0	150	138	92
0	150	137	91
0.01	148	129	97
0.1	147	108	44
0.4	139	15	32
1.0	141	9	21
2.0	143	1	22
1.0 + 0.5 γ biotin	142	134	86

* In colorimeter turbidity units

synthetic medium described by Badger (10), to which 20 per cent of beef heart infusion broth was added. In this medium the growth of the organism was better than in either the synthetic medium or the infusion broth alone. For the inoculum, 0.2 cc. of an 8 hour culture in blood-broth medium was transferred to 5 cc. of the semisynthetic medium and incubated for 7 hours. 0.2 cc. of this culture was used as the inoculum per 5 cc. of medium, which contained varying amounts of imidazolidonecaproic acid. The growth after 12 hours was measured in a Klett-Summerson colorimeter and is expressed as colorimeter turbidity units. The results of one such experiment are recorded in Table II. In this medium, which was found by microbiological assay to contain 0.005 γ of biotin per tube, imidazolidonecaproic acid inhibited the growth of types II and III pneumococci under these conditions, but had little effect on type I. Whenever this compound

inhibited the growth of pneumococci, the addition of larger amounts of biotin prevented the inhibition.

Imidazolidonecaproic acid inhibited the growth of *Escherichia coli* when grown in the synthetic medium described by Kalmanson and Bronfenbrenner (11). Biotin again nullified this inhibition.

Avidin-Combining Ability of Imidazolidone Aliphatic Acids—In conformity with the hypothesis that the urea grouping of biotin is essential for the combination of biotin with avidin (12), the yeast growth activity of desthiobiotin was also inhibited by avidin (7). Since a reproducible stoichiometrical relationship exists between avidin and biotin (13), it was possible to test various compounds for their ability to block the interaction between avidin and biotin. Since a given amount of avidin can remove a definite amount of biotin, as measured by diminished yeast growth, it should follow that a compound which can combine with avidin will be able to block, to a certain degree, the combination of biotin with avidin, leaving more biotin for yeast growth. On this basis, the imidazolidone aliphatic acids were tested for their ability to combine with avidin by adding them to tubes containing avidin, allowing a certain time interval for interaction, and then adding biotin to the mixture. The yeast growth in the tubes containing the imidazolidone aliphatic acid, avidin, and biotin was then compared with the growth in tubes containing only avidin and biotin. It was thus possible to determine the increase in yeast growth, and hence the available biotin, in the tubes containing the various imidazolidone aliphatic acids. Preliminary experiments of this type have indicated that the avidin-combining activities of the imidazolidone aliphatic acids vary in the same manner as their antibiotin activities, imidazolidonecaproic acid having the strongest affinity for avidin and imidazolidonevaleric acid the weakest.

DISCUSSION

It is of interest to note that in each type of activity so far demonstrated for the imidazolidone aliphatic acids imidazolidonecaproic acid is the most active. Imidazolidonecaproic acid has the same length side chain as desthiobiotin and differs from it only in the absence of the terminal methyl group. Shortening the aliphatic acid side chain to valeric acid markedly reduces the antibiotin and avidin-combining activity of the imidazolidone aliphatic acid.

Imidazolidonevaleric acid, which had been recrystallized several times, originally stimulated the growth of yeast (2). The small amount of yeast growth-promoting activity (0.0017 per cent that of biotin) of this compound made one suspect contamination. Since the imidazolidonevaleric acid did not stimulate the growth of *Lactobacillus casei*, its activity

could not be due to biotin. However, if biotin contamination arose somewhere in the synthetical steps prior to the treatment with Raney's nickel catalyst, any trace of biotin would be converted to desthiobiotin, which might then be retained as a contaminant in the imidazolidonevaleric acid preparation. Since recrystallization did not lower the yeast growth-promoting activity of the imidazolidonevaleric acid, it was cleaved to the corresponding diaminoheptanoic acid sulfate and was resynthesized from the latter with phosgene. This resynthesized preparation of imidazolidonevaleric acid was inactive as a stimulant for yeast growth. At high concentrations (2 to 4 mg. per 7 cc.), imidazolidonevaleric acid slightly inhibited the growth of both *Lactobacillus casei* and *Saccharomyces cerevisiae*.

Lengthening the aliphatic side chain of the imidazolidone aliphatic acid to heptanoic or octanoic acid likewise progressively diminishes the anti-biotin and avidin-combining activities, but not nearly to the same extent as shortening the chain by 1 carbon.

These data showing that imidazolidone aliphatic acids have the ability to interact with avidin further emphasize that the cyclic urea grouping is important in the biotin-avidin relationship.

The fact that imidazolidonecaproic acid does not support growth in the absence of biotin might indicate that *Saccharomyces cerevisiae*, strain 139, cannot introduce the methyl group to form desthiobiotin under these conditions. When from 0.5 to 10 γ of imidazolidonecaproic acid are added to yeast cultures which contain 2×10^{-4} γ of biotin per 7 cc., the growth of yeast is greatly stimulated above the growth obtained from 2×10^{-4} γ of biotin alone. The curves of Fig. 1 illustrate this effect. 2×10^{-4} γ of biotin stimulated yeast growth to a level of 101 colorimeter turbidity units, whereas with 1 γ of imidazolidonecaproic acid present in addition to the biotin the yeast growth reached a level of 155 colorimeter turbidity units. This amount of yeast growth is equivalent to 3.7×10^{-4} γ of biotin, although only 2×10^{-4} γ were added. Whether this increase in yeast growth stimulation is due to synthesis of desthiobiotin from imidazolidonecaproic acid remains to be determined. The relative increase in apparent biotin activity due to added imidazolidonecaproic acid is greater when the yeast grows in an optimum growth medium than when it grows in a more deficient medium. The increased biotin activity of a given amount of biotin due to added imidazolidonecaproic acid could also be the result of better utilization of the biotin present.

SUMMARY

A series of four imidazolidone aliphatic acids has been found to be antibiotics for *Saccharomyces cerevisiae*, Fleischmann strain 139, and

Lactobacillus casei. Imidazolidonecaproic acid was the most potent of this series, while imidazolidonevaleric acid was the least active. As the side chain was lengthened from caproic acid to heptanoic and octanoic acids, the antibiotin activity decreased. The antibiotin activity was counteracted in every case by the addition of biotin.

Preliminary experiments have indicated that the avidin-combining abilities of the imidazolidone aliphatic acids vary in the same manner as their antibiotin activities.

Imidazolidonecaproic acid also inhibited the growth of types II and III pneumococci and *Escherichia coli*.

The imidazolidone aliphatic acids were much more potent inhibitors of yeast growth when desthiobiotin was the growth stimulant than when biotin was the stimulant.

The authors are indebted to Mrs. Glenn Ellis, Miss Rachel Jewett, and Mrs. Margaret Sherwood for carrying out the microbiological tests.

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MICROBIOLOGICAL DETERMINATION OF LYSINE IN PROTEINS AND FOODS

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Microbiological methods for the assay of lysine (1-10), including the use of decarboxylase (3, 7) and a mutant of *Neurospora* (8), have been reported by several investigators.

In the work now presented, it was found that when the same medium and organism (*Lactobacillus arabinosus* 17-5) as previously employed for methionine assay (11) were applied to lysine a satisfactory curve could not be developed because of high blank values. That these values were not caused by traces of lysine associated with any of the other amino acids in the basal medium was shown by the fact that satisfactory blanks were obtained when *Leuconostoc mesenteroides* P-60 (2) was used. Our failure to obtain a satisfactory curve with *Lactobacillus arabinosus* is ascribed to an interrelationship between lysine and pyridoxine as shown by Stokes and Gunness (12) and Snell (13).

The standard curve employed for the lysine assays was prepared by using *Leuconostoc mesenteroides* and the same medium previously used for methionine (11), with the exception that the amount of L-arginine was increased from 96 to 416 mg.

EXPERIMENTAL

Leuconostoc mesenteroides P-60¹ was used in the assays described in this paper.

Basal Medium.—The basal medium was the same as that described in a previous paper for methionine (11), with the exception that the amount of L-arginine was increased as noted above. This increase was necessary because it raised the titration values at the higher levels of lysine. Below a level of 40 γ , however, the increase had no effect.

Assay Procedure.—The procedures followed for the cultures, inoculum, and preparation of samples were identical with those described for the assay of methionine with *Lactobacillus arabinosus*, with the exception that 0.05 N sodium hydroxide was used in the titration instead of 0.10 N. The

¹ Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C.

lower concentration was employed because of the smaller amount of lactic acid produced by *Leuconostoc mesenteroides*.

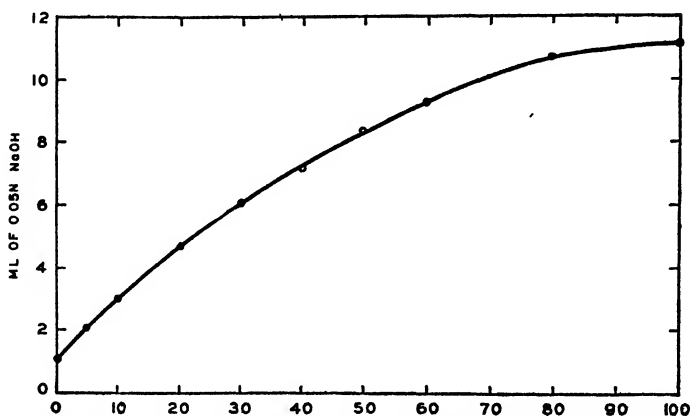


FIG. 1. Lysine curve The abscissa scale represents micrograms of L-lysine

TABLE I
Recovery of Lysine Added to Protein Hydrolysates

Protein hydrolysate*	Lysine				
	In hydrolysate*	Added	Total	Found	Recovery
	γ	γ	γ	γ	per cent
Gelatin	3.75	50.00	53.75	55.00	102
	7.50	50.00	57.50	60.00	105
	11.25	50.00	61.25	62.50	102
	15.00	50.00	65.00	67.00	103
Egg albumin	5.93	10.00	15.93	15.0	94.3
	11.86	10.00	21.86	22.5	103
	17.79	10.00	27.79	27.5	98
	23.72	10.00	33.72	33.5	99
Edestin	3.01	10	13.01	12.50	96
	3.01	20	23.01	22.50	98
	3.01	30	33.01	33.50	101
	3.01	40	43.01	43.30	101
	3.01	50	53.01	53.00	100

* Uncorrected for moisture and ash.

Preparation of Lysine Standards—L-Lysine solutions ranging in concentration from 5 to 100 γ per ml. were used in preparing the standard curve (Fig. 1). The titration values on this curve were not altered by the addition of 1.2 mg. of any of the nineteen amino acids to the medium.

Recovery of lysine added in different proportions to hydrolysates of

gelatin, egg albumin, and edestin gave results well within the experimental error for this type of assay (Table I).

TABLE II

*Lysine Content of Some Proteins and Foods Determined at Different Assay Levels**

Protein assay level	Lysine found									
	Whole wheat		Whole rye		Whole corn		Phaseolin		Cottonseed flour	
γ	γ	per cent	γ	per cent	γ	per cent	γ	per cent	γ	per cent
100							6 50	6 50		
200							13 00	6 50	5 30	2 65
300							19 50	6 50		
400							25 70	6 43	10 60	2 65
500	2 20	0 44	1 30	0 26						
600									15 00	2 50
700										
800									20 00	2 50
900										
1,000	4 00	0 40	2 50	0 25						
1,500	6 00	0 40	4 00	0 27						
2,000	8 00	0 40	5 50	0 27	7 00	0 35				
2,500	11 30	0 15	6 50	0 26	9 50	0 38				
3,000	21 50	0 43	14 50	0 29	19 50	0 39				
7,500	32 50	0 43	22 00	0 29	29 00	0 30				
10,000	42 00	0 42	30 00	0 30	36 80	0 37				
Average		0 42		0 27		0 37	6 45		2 58	

* Uncorrected for moisture and ash

TABLE III

Reproducibility of Lysine Content When Determined by Separate Assays

Material	Assay 1	Assay 2	Assay 3	Average
	per cent	per cent	per cent	per cent
Arachin	2 30	2 50	2 20	2 33
Conarachin	4 50	4 40	4 11	4 44
Whole wheat	0 41	0 43		0 42
" rye	0 34	0 38		0 36
Peas, black eyed	1 49	1 55		1 52
Egg albumin	5 73	5 93		5 83

* Figures not corrected for moisture and ash

Table II shows values found for phaseolin and several foods determined at different assay levels. Data on reproducibility of the amount of lysine found in a number of materials when determined by separate assays are given in Table III

TABLE IV

Lysine Content of Some Proteins and Foods (Percentages Calculated for Ash- and Moisture-Free Material)

Material	N	Lysine	Values from literature
	<i>per cent</i>	<i>per cent of food</i>	
Arachin	18.30	2.72	2.4 (14), 4.5 (14), 1.83 (15)
Casein	16.07	8.20	8.3 (2), 8.1 (3), 7.7 (4), 7.6 (5), 7.6 (6), 7.7 (7), 8.3 (10), 5.4 (16), 8.7 (17), 6-8 (18)
Conarachin	18.20	4.69	7.0 (14)
Cottonseed globulin	18.00	4.15	4.90 (19), 2.25 (20)
Coconut globulin	17.42	4.37	
Edestin	18.55	3.23	2.32 (14), 3.90 (21)
Ovalbumin (crystalline)	15.98	6.32	6.6 (4), 6.0 (5), 6.3 (8), 5.0 (14)
Gelatin (Bacto)	18.32	4.44	5.8 (4), 4.1 (6), 6.9 (8), 5.04 (14)
Glycinin	17.30	6.90	
Lactalbumin	15.39	10.80	8.4 (6), 9.6 (14), 9.87 (22) 11.4 (23)*
Peanut, total globulins	18.01	3.50	
Phaseolin (navy bean)	16.07	7.20	
Ox muscle...	16.00	10.00	7.9 (6), 8.1 (24)
Wheat bran globulin	17.76	4.15	
Zein	16.00	0.21	0.06 (3), 0.00 (14)
Barley, pearled	1.86	0.28	0.26 (9)
Brazil nut meal	9.03	1.80	
Corn, whole yellow	2.22	0.32	0.44 (9), 0.40 (14), 0.35 (14), 0.14 (25)
“ germ, defatted	3.93	1.45	1.41 (14)
Cottonseed flour	10.36	2.92	2.42 (9), 1.76 (14)
Egg, whole, dried	8.11	4.00	3.04 (14)
Milk, dry, skim	6.57	2.91	3.57 (4), 2.90 (9), 3.41 (10), 3.11 (14)
Oatmeal	2.73	0.51	0.57 (14), 0.22 (26)
Peanut flour	10.15	1.92	1.91 (14), 1.93 (14)
Peas, black-eyed	4.15	1.70	
Rice, white	1.26	0.22	0.34 (27), 0.24 (27)
Rye, whole	1.98	0.41	0.51 (4), 0.41 (9)
Soy bean flour	8.85	3.97	3.72 (3), 3.01 (4), 3.72 (9), 3.01 (14)
Wheat, whole	3.07	0.48	0.54 (4), 0.47 (9), 0.51 (14)
“ germ, defatted	6.50	2.52	2.24 (14), 1.13 (28)
Yeast, dried, brewers'	7.71	3.50	3.08 (4), 3.08 (14)

* For β -lactoglobulin by isotope dilution method

The lysine values (Table IV) found for the proteins and foods² assayed agree quite well with those reported for the same materials by others using different microbiological methods. Considerable discrepancy exists, however, between several of them and those determined by chemical procedures. The values now reported have been corrected for moisture and ash. Although the ash content of the isolated proteins is low, that of the foods, however, is considerable, as illustrated in Table V.

Values reported for the same proteins by different workers are not always comparable because of differences in nitrogen content of the proteins assayed. Since the nitrogen content of the proteins is not given in many instances, it is not possible to correct the lysine values to the nitrogen content given in the second column of Table IV. For example, values for casein were determined on Labco casein, a product varying in nitrogen

TABLE V
Moisture and Ash Content of Some Foods

Food	Moisture	Ash
	<i>per cent</i>	<i>per cent</i>
Brazil nut meal	7.01	9.77
Corn germ	9.47	7.96
Cottonseed flour	6.41	5.99
Egg, whole, dried	7.18	2.92
Milk, dry, skim	5.99	7.42
Soy bean flour	5.07	6.12
Wheat germ	6.76	4.75
Yeast, dried, brewers'	6.82	4.71

content within a rather wide range. If the nitrogen contents were given, the lower values for lysine might well agree more closely with the values here reported when recalculated to an ash- and moisture-free basis and 16 per cent nitrogen content. In the case of foods, when the nitrogen content was given, the lysine values have been corrected to the nitrogen content shown in Table IV.

SUMMARY

A microbiological method is described for the determination of lysine in proteins and foods with *Leuconostoc mesenteroides*. The results of assays on thirty-one proteins and foods agree closely with those obtained on the same materials by other microbiological methods.

² The sources and preparation of the samples assayed are given in a previous publication on the determination of methionine (29)

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BIOCHEMISTRY OF THE SPHINGOLIPIDES*

I. PREPARATION OF SPHINGOLIPIDES FROM BEEF BRAIN AND SPINAL CORD

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Nerve tissue contains a high concentration of lipid material of an extremely complex nature. Among the lipid constituents there are at least three, the cerebroside (1), sphingomyelins (1), and gangliosides (2-5), which are derivatives of the organic base sphingosine. Sphingosine also may be present in other compounds (6). As a matter of convenience it is proposed that the term sphingolipide be used to designate these substances.

Several years ago we reported preliminary data indicating that the currently accepted formula for sphingosine was probably incorrect (7). These results will be described in greater detail later. It is the purpose of this paper to present the methods we have found most suitable for the preparation of large quantities of sphingolipide.

The only practical source of the sphingolipides is brain or spinal cord, although they occur in blood, liver, kidney, spleen, and other organs in small amounts. Brain is reported to contain 2.5 to 3.0 per cent of cerebroside (8-11) and 1.0 to 1.3 per cent of sphingomyelin (9, 12, 13). There is little specific information in the literature concerning the lipid composition of spinal cord. Fränkel and Linnert (14) indicated that beef spinal cord contains only 66 per cent water and that 80 per cent of the solid material is lipid in nature; each of these observations is encouraging from a preparative standpoint. However, Schuwirth (15) reported that human spinal cord contains 75 per cent of water and only 1.3 and 0.7 per cent respectively of cerebroside and sphingomyelin. At any rate, it seemed desirable to investigate both brain and spinal cord as a possible source of sphingolipide.

A variety of methods have been described for isolating sphingolipides

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from nerve tissue but practically all involve (1) dehydration of the fresh tissue, (2) extraction of the glycerophosphatides, and (3) extraction of the cerebrosides and sphingomyelins, either singly or in one fraction. Dehydration of the tissue has usually been accomplished with acetone (16-18) or ethanol (19-21). Cholesterol, neutral fat, and small amounts of other lipides are also removed by acetone. Lecithin and cephalin have been extracted with petroleum ether (18, 22-24) or ether (8, 16, 20, 25), in which the sphingolipides are relatively insoluble. The residue is a light brown, friable, reasonably stable powder.

A variety of solvents have been employed in extracting the sphingolipides from this material. Hot ethanol (85 per cent (8, 16, 19) or absolute (20, 26, 27)) removes both cerebrosides and sphingomyelins. Klenk (18) employed hot acetone in a continuous extraction apparatus (the solubility of sphingolipides in this solvent is small). Rosenheim (22) removed the cerebrosides with cold pyridine and the sphingomyelins with hot pyridine. Page (24) proposed the use of tetralin as the extracting agent. Levene (26) preferred an alternative method in which dehydrated beef brain was extracted directly with hot alcohol. The precipitate obtained on cooling the extracts (so called "white matter" or "protagon") was treated with ether to remove lecithin and cephalin.

We have tested various combinations of these procedures in working up several hundred pounds of fresh beef brain and spinal cord. Acetone is the most convenient dehydrating agent. It is essential that the tissue be thoroughly desiccated, since the solubility of sphingolipides in ether or petroleum ether (21) is greatly increased by small amounts of water. Ether is somewhat more satisfactory than petroleum ether for removing lecithin and cephalin. Sphingomyelin and some cerebrosides are also extracted and may be partially recovered by cooling and centrifuging the ether solutions. However, it was discovered that if the ether extraction is carried out below 25° the loss is small and it is not economical to attempt recovery.

In our hands neither pyridine or tetralin proved to be satisfactory solvents for extracting the sphingolipides. With tetralin the yield was poor. Pyridine gave a somewhat better yield but it is an inconvenient and expensive solvent for large scale work. The use of ethanol proved highly satisfactory (95 per cent is superior to 85 per cent), giving excellent yields of good quality product. By the procedure finally adopted 45.4 kilos (100 pounds) of fresh beef brain gave 11 to 12 kilos of acetone-extracted material, 6.5 to 7.5 kilos of acetone-ether-extracted powder, and 1300 to 1600 gm. of crude sphingolipide, which still contained some lecithin and cephalin. These results agree reasonably well with those of Rosenheim (22), Klenk (18), and Loening and Thierfelder (25). Calculated on a cerebroside content of 3 per cent and a sphingomyelin content of 1.2 per cent, 45.4 kilos of

fresh beef brain should give 1906 gm. of sphingolipide. The ether extracts presumably contain the major portion of the unrecovered material.

In processing spinal cord by this method, surprisingly better results were obtained. The yield of crude sphingolipide from 45.4 kilos of spinal cord ranged from 2200 to 2500 gm., compared to 1300 to 1600 gm. with brain. Furthermore, the spinal cord product was an almost white, crisp solid, whereas the material from brain was light brown and less granular. Complete cerebroside and sphingomyelin analyses on the various fractions from a 45.4 kilo run of spinal cord showed that the 2582 gm. of crude sphingolipide obtained consisted of 1838 gm. of cerebroside, 606 gm. of sphingomyelin, and only 138 gm. of other material. For our purposes spinal cord has still another advantage over brain. The sphingolipides obtained from spinal cord can be used directly in the preparation of sphingosine, whereas crude sphingolipide from brain must be recrystallized from glacial acetic acid to remove material which otherwise decomposes during the hydrolysis and interferes with the isolation of sphingosine.¹

The procedure described below is similar to that of Loening and Thierfelder (25) and represents the experience acquired in processing 600 pounds of brain and 2000 pounds of spinal cord.

EXPERIMENTAL

Preparation of Tissue—Beef brain and spinal cord were obtained through the generous assistance of Dr. Porsche of Armour and Company. They were removed in the afternoon, frozen, and shipped so as to reach our laboratories the next morning. In this way we were assured of having fresh material. In the early part of our work, the tissues were thawed, peeled, and ground in a small laboratory mill. It was subsequently found that a 100 pound lot of frozen material could be ground directly to a soft paste in a commercial sausage grinder. This process is much more convenient, especially for large scale preparative work.

Since both brain and spinal cord were worked up in the same manner, only one typical experiment will be described.

Dehydration with Acetone—100 pounds of ground spinal cord were placed in two 10 gallon crocks and extracted overnight with 40 liters of acetone. The mixture was stirred occasionally. The acetone was removed as completely as possible by decantation and the extraction was repeated in the same way with 20 liters of acetone. A total of six extractions was made.

¹ It seems probable that this material consists mainly of gangliosides which are present in brain (28) but not in spinal cord (15), and which are unstable toward vigorous acid treatment. Our crude spinal cord sphingolipide contained only a trace of ganglioside as measured by the orcinol test (29) for neuraminic acid, a constituent of the gangliosides.

By the fourth extraction, the majority of the water had been removed and the product had become more flocculent. In the last three extractions the acetone was removed by filtration. These extracts contained large quantities of cholesterol and only small amounts of water.² The final acetone-extracted material was a light brown, gummy solid, weighing 11.8 kilos (range 9 to 12 kilos for spinal cord, 8.5 to 12 kilos for brain).

Extraction of Glycerophosphatides The acetone-extracted residue was divided between five 12 liter flasks and extracted for 18 hours with 20 liters of commercial dry ether. The flasks were cooled to 20–25° under the tap and the mixture was stirred occasionally. Filtration of the extraction mixture was not practicable, since evaporation of the ether resulted in the deposition of phospholipides on the filter. To avoid this difficulty a double layer of cheese-cloth was tied over the neck of the flask around a breather tube extending to the bottom of the flask. The flasks were then inverted and as much ether as possible was drained off. After the third ether extraction sufficient phospholipide had been removed to make filtration possible. Five ether extractions³ were usually made although some glycerophosphatide still remains in the residue at this point.

The ether-extracted residue was air-dried, giving 5.7 kilos (range 5.2 to 6.0 kilos for spinal cord, 6.0 to 7.5 kilos for brain) of a light brown friable material, which was moderately stable on exposure to air and could be stored almost indefinitely for use in the final step.

Extraction of Sphingolipides—The acetone-ether-extracted material (5.7 kilos) was divided equally among four 12 liter flasks and extracted with 20 liters of boiling ethanol (95 per cent) for 10 to 15 minutes. The mixture was filtered on a large Büchner funnel and the extraction repeated three more times. The ethanol filtrates were cooled overnight in an ice box and the precipitated sphingolipides were removed by filtration, washed once with acetone, and air-dried. The product was a white powdery material weighing 2275 gm. (range 2000 to 2500 gm for spinal cord, 1300 to 1600 gm. for brain).

In view of the few data in the literature on the composition of the sphingolipides of spinal cord, analyses were made on each of the fractions from a 100 pound portion which was processed by the above procedure with the following exceptions. The ether extractions were made at room temperature and each of the extracts was cooled to 0° and the precipitated material

² The acetone was recovered from the extracts by saturating them with salt, separating the supernatant liquid, and distilling it through a fractionating column. The last three extracts were worked up separately from the first.

³ The ether extracts on cooling in an ice bath deposited material containing sphingolipides. However, the yield was not large and the product was brown and somewhat sticky. For preparative work it is more economical to discard the ether extracts.

removed by centrifugation. The ethanol-extracted residue was further extracted with 15 volumes of 1:1 methanol-chloroform at room temperature. This solution was concentrated under reduced pressure and the residue was washed several times with acetone, giving a brown, somewhat sticky powder. Each of the lipide fractions thus obtained was analyzed for cerebroside, sphingomyelin, nitrogen, and phosphorus. Cerebroside was determined by the method of Brand and Sperry (30) with the galactose procedure of Miller and Van Slyke (31), sphingomyelin by the method of Thannhauser and coworkers (32-34) as modified by Erickson *et al.* (35). The results of this study are summarized in Table I. The total yield of cerebroside was 4.2 per cent of the fresh weight of the spinal cord; the yield

TABLE I
Composition of Lipide Fractions Obtained from 45.4 Kilos of Fresh Beef Spinal Cord

Extract	Weight		Cerebroside		Sphingomyelin		Total N	Total P
	gm.	per cent	gm.	per cent	gm.	per cent	per cent	per cent
Ether 1	335.8	59.5	200	30.3	102	2.06	1.43	
" 2	142.8	52.5	75	33.7	48	2.09	1.61	
" 3	79.1	67.5	53	35.2	27	2.08	1.54	
" 4	40.5	58.6	24	33.6	14	2.17	1.37	
Ethanol 1-3	1118.7	74.1	1051	20.4	290	2.11	0.98	
" 4	270.7	85.0	230	18.0	49	2.00	0.69	
" 5	136.2	73.5	100	14.0	19	2.13	0.62	
" 5a*	158.7	66.3	105	36.2	57	2.20	1.34	
Methanol-chloroform	215.0	31.8	68	8.6	18	2.32	1.69	
Ether, total	598.2		352		191			
Ethanol, total	1984.3		1486		415			
Grand total	2797.5		1906		624			

* This fraction was obtained by concentrating and cooling the combined ethanol filtrates.

of sphingomyelin was 1.37 per cent. The high cerebroside content of the ethanol fractions makes this material especially suitable for the preparation of sphingosine. The methanol-chloroform extraction removed only a small amount of poor quality material and is not a practical process for preparative work.

SUMMARY

A study was made of the various procedures reported for the isolation of cerebroside and sphingomyelin from nerve tissue. By the procedure finally adopted 100 pounds of fresh beef brain yielded 1300 to 1600 gm. of a crude sphingomyelin-cerebroside mixture; 100 pounds of beef spinal cord gave 2200 to 2500 gm. The latter material is more satisfactory for the prepara-

tion of sphingosine. Cerebroside and sphingomyelin analyses are reported for the lipid fractions from 100 pounds of spinal cord.

It is proposed to designate those lipides derived from sphingosine as sphingolipides.

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STUDIES ON THE REACTION OF CERTAIN CAROTENOIDS WITH ANTIMONY TRICHLORIDE*

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Since both carotene and vitamin A yield a blue color with SbCl_3 , attempts to determine the vitamin quantitatively in a mixture depend, among other things (1, 2), upon the accuracy with which the blue color due to carotene can be estimated. Values published for the blue equivalent of carotene range from 3.5 to 13, calculated as micrograms of apparent vitamin A per 100 γ of carotene (3). This discrepancy does not result in serious errors in the analysis of mixtures relatively rich in vitamin A, but in materials such as eggs, concentrates for animal feeding, or aliquots of mixed human diets the blue color from carotenoids may constitute a major fraction of the total absorption, and hence the proper evaluation of this component becomes critical. The color due to vitamin A reaches maximum intensity in 2 to 5 seconds after the addition of a SbCl_3 reagent (4), whereas that from the carotenoids forms more slowly (5-7) and varies with the pigment. Concentrates from summer butter have been observed to react more slowly than those from winter butter (8). The present study deals with variations in the intensity and rate of formation of the blue color when SbCl_3 reacts with derivatives of carotene. It has been recognized that extraction and saponification procedures normally employed in analyses may result in either an oxidation or isomerization of the pigment (9-12).

Methods

Solutions of carotene from various sources were analyzed for both carotene and apparent vitamin A by standard colorimetric procedures. Determinations were made on fresh material and on carotene that had been exposed to oxidation or to isomerization. The pigment was dissolved in 10 ml. of redistilled chloroform and the intensity of light was measured through a 440 $\text{m}\mu$ filter in an Evelyn photoelectric colorimeter. The galvanometer readings were converted to micrograms of carotené by multiplying the L values by the factor 3.35. For the determination of blue values the

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solutions were treated with SbCl_3 , as in the determination of vitamin A (4), and a drop of acetic anhydride was added routinely to all tubes to prevent turbidity. Galvanometer readings were measured through a $620\text{ m}\mu$ filter at intervals of 5 to 10 seconds and the apparent vitamin A was calculated from the maximum intensity of the blue color attained by multiplying the appropriate L values by 1.33 (13). Thus the apparent vitamin A for any carotenoid represented the correction factor that would have to be applied to the total blue color in a determination of vitamin A in mixtures.

EXPERIMENTAL

Blue Color from Carotene or Carotene Fractions from Crude Materials—Carotenoids from fresh vegetables were extracted repeatedly with 95 per cent ethanol in a Waring blender; dried feeds were extracted with a mixture of ethanol and Skellysolve B (b.p. $64-68^\circ$). The extracts were saponified with alcoholic KOH, the pigment was transferred to petroleum ether (Skellysolve B or Skellysolve A (b.p. $34-37^\circ$)), and the xanthophylls were removed with 85 per cent ethanol. The petroleum ether solution was then evaporated to dryness under a vacuum without heating and the pigment taken up in CHCl_3 .

On treatment with SbCl_3 the pure carotene developed a maximum color within 10 seconds and then faded rapidly. The apparent vitamin A value of the solution was 5.7 per 100 γ of carotene (Table I). The carotene fraction from carrot roots developed a blue color at a rate similar to that of carotene itself (Table I). All other natural materials analyzed, however, whether fresh or dried, yielded carotene fractions with abnormal rates of color formation; the maximum intensity was reached only after 15 to 120 seconds. Moreover, for all of the crude carotene solutions, including those from carrot root, the blue to yellow values were usually double those for solutions of pure carotene, and in some fractions, *e.g.* those in which oxidation had taken place, the values rose to 52.4 (Table I). The abnormal character of the SbCl_3 reaction of the crude materials was not altered by saponifying with 15 per cent KOH in ethanol for 30 minutes under a reflux.

Reaction between Oxidized Carotene and SbCl_3 —Commercial carotene, containing approximately 15 per cent of α - and 85 per cent of β -carotene, was dissolved in chloroform or Skellysolve B, and aliquots were permitted to evaporate in the air and light of the laboratory for various lengths of time (Table II). Changes in both the yellow and blue values were determined as before. On exposure to air the intensity of the yellow color decreased rapidly; 40 per cent had disappeared within 2 days and 64 per cent by 4 days. The chromogenic equivalent, however, decreased much less rapidly; the loss in apparent vitamin A was only 14 per cent after 2 days and 27 per cent after 4 days (Table II). The blue to yellow ratio (micrograms of

apparent vitamin A per 100 γ of carotene) therefore increased markedly. The rate at which the blue color developed was also abnormal (Table II), with the more highly oxidized samples 60 seconds were required for maximum intensity, and the color was stable for a long time thereafter. The rate of oxidation of carotene crystals seemed to depend somewhat upon the previous treatment of the carotene. A carotene solution that had been stored in chloroform for 13 days at 0° deteriorated much more rapidly on evaporation than ones previously stored in petroleum ether (14).

TABLE I
Characteristics of Blue Color Formed with SbCl₃ and Carotene Fraction of Certain Plant Materials

Source of carotene fraction	Apparent vitamin A per 100 γ carotene	Rate of color formation, maximum intensity
	γ	
β -Carotene	5.7	5-10 sec., fades rapidly
Fresh carrots*	12.0	Immediately, fades rapidly
Cooked " *	16.6	" " "
Dried " *	11.5	" " "
Carotene from fresh carrots oxidized 1 day*	44.5	" " after 2 min
Fresh carrots	29.4	" " rapidly
Cooked "	13.4	" " "
Dried "	12.9	" " "
Carotene from fresh carrots oxidized 1 day	52.4	" stable over 2 min
Carotene from cooked carrots oxidized 1 day	18.3	After 1 min., faded slowly
Egg mash	23.1	" 2 "
Alfalfa meal, dried, 300°	8.9	" 30 sec., faded slowly
" " sun-dried	18.7	" 90 " " "
" " vacuum-dried	12.3	" 30 " " fairly rapidly
Fresh chard	7.6	" 15-30 sec., faded slowly
" alfalfa	9.3	" 15-30 " " "

* The carotene extract was not saponified.

A separation of unchanged carotene from its oxidation products was effected by treating mg. portions of commercial carotene that had been oxidized for 24 hours at room temperature with 100 ml. of 80 per cent methanol, and warming slightly to facilitate solution. After cooling, the insoluble portion was removed by filtration in a sintered glass funnel and was washed with 80 per cent methanol. Both the precipitate and filtrate were then transferred to chloroform, and yellow and blue determinations were made as before. The precipitate showed a blue to yellow ratio, 6.05,

very similar to that for pure carotene, 5.6, and the rate of color formation was also that of carotene itself. The fraction soluble in 80 per cent methanol, on the other hand, developed a blue color at an abnormal rate, with a value of 20.4 γ of apparent vitamin A per 100 γ of carotene.

In order to determine whether any component of commercial carotene was responsible for anomalous Carr-Price reactions, the carotene was adsorbed out of Skellysolve B solution onto a column consisting of 3 parts of $Ca(OH)_2$ and 1 part of Hyflo Super-Cel. The developing solvent was Skellysolve B. Three fractions resulted. Fraction 1 consisted of a firmly bound layer near the top of the column which moved only very slowly, but

TABLE II
Reaction between Oxidized Carotene and $SbCl_3$

Treatment of carotene	Carotene	Apparent vitamin A	Apparent vitamin A per 100 γ carotene	Rate of color formation, maximum intensity
	γ per ml.	γ per ml.	γ	
Fresh chloroform solution	199.0	11.4	5.7	10 sec., fades for 2 min.
Oxidation of crystals*				
1 day, 20°	131.2	11.5	8.7	15 " stable " 2 "
2 days, 20°	119.5	9.8	8.3	30-60 sec., stable 2 min.
3 " 20°	103.3	9.9	9.6	30-60 " " 2 "
4 " 20°	71.9	8.3	11.6	60 sec., stable 2 min.
Chloroform solution stored 13 days, 0°	188.0	10.7	5.7	10 " fades slowly
Oxidation of crystals*				
1 day, 20°	123.6	11.5	9.3	2-5 min., stable
2 days, 20°	92.6	10.6	11.6	5-15 " "
3 " 20°	38.8	8.3	21.4	5-15 " "
4 " 20°	17.6	6.9	30.2	5-15 " "

* The oxidation was carried out by permitting the solvent to evaporate, and the crystals to be exposed to the air and light of the laboratory.

which could be developed into four or five minor bands by treatment with Skellysolve B containing 3 to 5 per cent of acetone. Most of these bands probably represented oxidation products. Fraction 2 was β -carotene; Fraction 3 was α -carotene. Either the main bands were eluted by the flowing chromatogram techniques or the zones were separated mechanically and the pigments eluted with a mixture of 5 per cent ethanol in Skellysolve B. The unknown pigments of Fraction 1 developed the Carr-Price color at an abnormal rate, and the blue to yellow ratio was also higher than that for α - or β -carotene (Table III). Similarly, when partially oxidized carotene was passed through $Ca(OH)_2$, the pigments responsible for the abnor-

mal reaction with SbCl_3 were found to accumulate at the top of the column (Table III).

Stereoisomers of Carotene—The abnormal reaction between the oxidation products of carotene and SbCl_3 raised the question whether stereoisomers of carotene might not also differ from the all-trans isomer in this respect. For the production of stereoisomers (12) solutions containing 0.2 mg. of rechromatographed β -carotene and 0.004 mg. of I_2 per ml. of petroleum ether were covered with a watch-glass and exposed for 1 hour to light from a 100 watt incandescent lamp at 20 cm. The solution was then washed four times with 0.1 M $\text{Na}_2\text{S}_2\text{O}_3$ and twice with distilled water. This crude isomerization mixture yielded approximately the same intensity of blue per unit of yellow as β -carotene itself, but the Carr-Price reaction was abnormal in that the

TABLE III
Characteristics of Blue Color Formation of Main Components of Commercial Carotene

Treatment of carotene	Apparent vitamin A per 100 γ carotene	Rate of color formation, maximum intensity
Fresh commercial carotene solution	5.6	10 sec., faded rapidly
Fraction 1	9.9	2 min., " slowly
2, β -carotene	5.25	10 sec., " rapidly
2, oxidized 1 day, 20°	11.6	2 min., " slowly
3, α -carotene	5.13	15 sec., " rapidly
3, oxidized 1 day, 20°	10.5	2 min., " slowly
Commercial, carotene oxidized 1 day, 20°	18.9	1 " stable 2 min
Fraction 1	20.5	5 " faded slowly
2, β -carotene	5.7	10 sec., " rapidly
3, α -carotene	5.1	15 " " "

blue color was stable for over 2 minutes in contrast to the rapid fading of the blue due to all-trans β -carotene (Table IV). Samples of β -carotene, illuminated and extracted as before but not exposed to iodine, yielded a normal Carr-Price reaction.

For the separation of the stereoisomers 50 mg. of crystalline β -carotene were isomerized, the solution was washed as before, and the resulting mixture chromatographed on a percolator type column 90 mm. (top) \times 270 mm. The adsorbent consisted of a mixture of 3 parts of $\text{Ca}(\text{OH})_2$ (Merek) and 1 part of Hyflo Super-Cel. The chromatogram was developed with a 0.5 per cent solution of acetone in Skellysolve B, with the separation of neo- β -carotene U, β -carotene, neo- β -carotene B, neo- β -carotene E, and several other unidentified minor bands. The bands were removed mechanically, and the pigments eluted from the adsorbent with a 5 per cent solution

of absolute ethanol in Skellysolve B. The various pigments were then re-chromatographed on a smaller column until they appeared homogeneous.

Solutions of the isomers in Skellysolve B exhibited the following maxima in the Beckman spectrophotometer.

Compound	Wave-lengths, in μ	Literature (12)
Neo- β -carotene U	482, 451	481, 450
" B.....	476.5, 445.5	475.5, 443.5
" E.....	478, 446	479.5, 447
Top firmly held bands ..	484, 453	481, 453

(The literature values given are for petroleum ether solutions.)

In general all of the stereoisomers of carotene reacted with SbCl_3 much like β -carotene itself, with relatively minor variations in the rates of color

TABLE IV

Effects of Isomerization of β -Carotene on Apparent Vitamin A to Carotene Ratio and on Carr-Price Reaction

Sample	Apparent vitamin A per 100 γ carotene	Rate of color formation, maximum intensity
	γ	
β -Carotene	5.61	5 sec., faded rapidly
Isomerized β -carotene (I_2 catalysis)	6.5	Immediately, stable over 2 min.
Treated as above, except I_2 not used	5.37	5 sec., faded rapidly
Top firmly held band from isomeric mixture	8.9	15-30 sec., faded slowly
Neo- β -carotene U	5.7	5-10 " " rapidly
" B	8.2	30-60 " " very slowly
" E	8.1	30-45 " " slowly

formation and in the blue to yellow ratios. Neo- β -carotene U was like all-trans β -carotene in both respects, but neo- β -carotene B formed a blue color that increased in intensity for 30 to 60 seconds after treatment with SbCl_3 and then faded very slowly, in contrast to the usual rapid formation of color and rapid fading of the natural isomer. Neo- β -carotene E and the mixture of isomers retained at the top of the column also reacted slowly with SbCl_3 . The blue to yellow ratio of most of the isomers ranged from 8.1 to 8.9 γ of apparent vitamin A per 100 γ of carotene, as compared to 5.6 for all-trans β -carotene (Table IV). Thus isomerization may be responsible for variations both in the amount of blue color formed and in the rate at which it forms or disappears. These variations are sufficient to introduce an element of uncertainty in the correction factor to be applied in the analysis of crude mixtures for vitamin A value, but it is also evident that as chromogens

the stereoisomers resemble β -carotene more closely than the oxidation products do. The highest blue to yellow ratio from the isomers, 8.9 (Table IV), is very much closer to the ratio for β -carotene, 5.6, than that of certain "carotene fractions" from carrots, 11.5:52.4 (Table I), or the ratios of 9.3:39.2 observed on oxidized commercial carotene (Table II). Meunier (15) has suggested that the blue derivatives of carotene are always in the all-trans form after reaction with SbCl_3 .

DISCUSSION

The wide variations in the blue to yellow ratios observed on carotene fractions from natural materials raise the question whether it is ever advisable to use such correction factors in the determination of vitamin A in crude mixtures. If vitamin A and pure carotene only were present, a satisfactory correction would be possible, but in crude materials oxidation often has taken place before the sample is submitted for analysis; and hence, any attempt to determine vitamin A becomes one of measuring the vitamin in the presence of several types of interfering agents, including carotene itself, the effect of which is constant and reproducible, and the oxidation products, which represent a variable component capable of exerting a much greater effect upon the SbCl_3 reaction, both qualitatively and quantitatively (Table III), than carotene itself. In addition, other carotenoids could exert variable effects on the reaction.

Accordingly it is almost imperative that these components be separated by chromatography, if reliable analyses for vitamin A are desired. Thompson *et al.* (16) have established conditions under which vitamin A from dehydrated eggs is distributed on Ca(OH)_2 between and including the zones due to β -carotene and cryptoxanthine. Under their conditions the more harmful oxidation products of carotene would be retained at the top of the absorption column.

SUMMARY

1. Under conditions ordinarily employed in the colorimetric determination of vitamin A, the blue from carotene reached maximum intensity in 5 to 10 seconds, and then faded rapidly. The blue from 100 γ of pure carotene equaled that from 5.6 γ of vitamin A. Carotene fractions from crude natural products formed blue colors that reached maximum intensity in 15 to 120 seconds with apparent vitamin A-equivalents ranging from 7.6 to 52.4 γ per 100 γ of carotene.

2. Partially oxidized carotene developed blue colors with SbCl_3 that increased in intensity for as long as 15 minutes after the addition of the reagent, with grossly abnormal blue to yellow ratios. The interfering agents could be separated from the carotene by extraction with 80 per cent methanol, or by chromatography.

3. Most stereoisomers of β -carotene reacted with SbCl_3 somewhat more slowly than the natural all-trans isomer, and the blue to yellow ratio was also increased slightly.

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THE UTILIZATION OF PHENYLALANINE AND TYROSINE DERIVATIVES BY MUTANT STRAINS OF *ESCHERICHIA COLI**

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The production of several mutant strains of *Escherichia coli* which require for growth an exogenous source of particular amino acids (1) has made possible a study of the manner in which individual mutants can use peptides and other amino acid derivatives for growth. In the present communication, data are presented on the utilization of derivatives of phenylalanine and tyrosine by appropriate mutants of *Escherichia coli*.

One of the mutant strains (No. 58-278) which requires phenylalanine was obtained by irradiation with x-rays of strain 58, a mutant strain obtained previously and characterized by a requirement for biotin (2). Two additional strains which require phenylalanine (strains 58-1899 and 58-6314) and a strain which requires tyrosine (strain 58-5030) were obtained upon exposure of strain 58 to methyl-bis(β -chloroethyl)amine (3). No differences could be observed among the three strains which were unable to synthesize phenylalanine from the constituents of the basal medium (2) and in the present discussion they will be considered as a single strain, *i.e.* *phenylalanineless*.

The amino acid requirement of each mutant strain was completely specific; the *phenylalanineless* strain grew in the presence of L-phenylalanine but not in the presence of L-tyrosine, and the *tyrosineless* strain grew in the presence of L-tyrosine but not of L-phenylalanine. For half maximal growth in 24 hours at 30° the *phenylalanineless* strain required, per 10 ml. of medium, approximately 1×10^{-4} mM of phenylalanine or 2×10^{-4} mM of DL-phenylalanine. The amount of L-tyrosine which gave, per 10 ml. of medium, half maximal growth for the *tyrosineless* strain was 0.9×10^{-4} mM and the amount of DL-tyrosine was 2.1×10^{-4} mM (Fig. 1). The D antipodes were not utilized for growth and, as may be seen from the data on the DL-amino acids, did not inhibit growth under the experimental conditions employed in this study.

In the study of the utilization of derivatives of phenylalanine and tyrosine, these were added to the basal medium, and, if growth occurred, a

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determination of the amount required for half maximal growth was carried out. Experiments were performed to determine any possible sparing action of test compounds in the presence of sufficient L-phenylalanine or L-tyrosine to permit half maximal growth. Under these conditions, none of the compounds tested exerted an inhibitory action on bacterial growth.

Utilization of Peptides of Phenylalanine and Tyrosine—As will be noted from Table I, glycyl-L-phenylalanine and L-phenylalanylglycine, on an equimolar basis, are approximately as effective in promoting growth as is L-phenylalanine, while L-glutamyl-L-phenylalanine is markedly less effective for growth. When the phenylalanine carboxyl is converted to an amide,

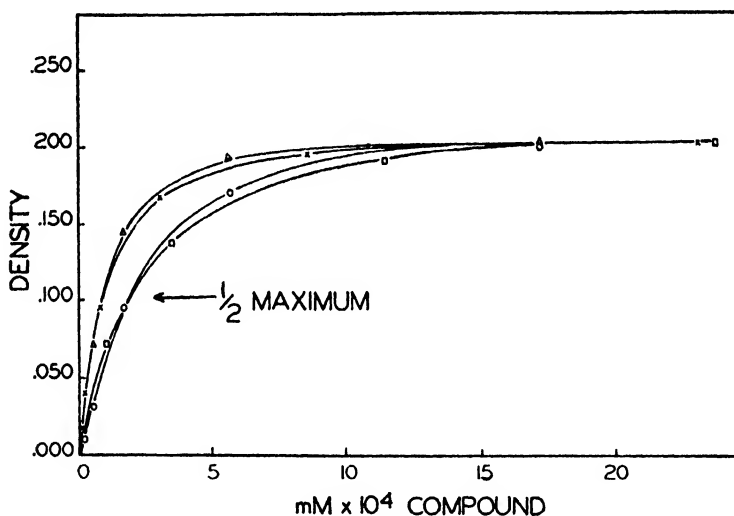


FIG. 1. Growth curves of mutants of *Escherichia coli* in the presence of added phenylalanine or tyrosine. *Phenylalanineless* strain; X, L-phenylalanine, O, DL-phenylalanine. *Tyrosineless* strain; Δ, L-tyrosine, □, DL-tyrosine.

as in L-phenylalaninamide and glycyl-L-phenylalaninamide, the ability of the resulting compounds to promote growth is greatly reduced. The fact that none of the compounds tested is more effective than L-phenylalanine itself suggests that these derivatives must be split to yield the free amino acid, which then serves as a growth factor. If this is the case, then the variation in the effectiveness of the individual phenylalanine derivatives may be interpreted as a reflection of the rate at which they are hydrolyzed by the bacterial enzymes.

The data in Table I also show that carbobenzyglycyl-L-phenylalanine has some growth-promoting activity which is, however, much less than that found for the free peptide.

It was of interest to compare the results on the utilization of phenylalanine peptides by the *phenylalanineless* mutant with those on the effect of analogous tyrosine peptides on the *tyrosineless* mutant. It will be seen from Table I that, in all cases tested, the comparable tyrosine peptides were effective in promoting growth. Furthermore, the quantitative data indicate that the relative efficacy with which a given peptide replaces its

TABLE I
Effect of Phenylalanine and Tyrosine Derivatives on Growth of Mutant Strains of Escherichia coli

Phenylalanineless strain		Tyrosineless strain	
Compound*	Concentration giving half maximal growth	Compound*	Concentration giving half maximal growth
	$mM \times 10^4$		$mM \times 10^4$
L-Phenylalanine	1	L-Tyrosine	0.9
DL-Phenylalanine	2	DL-Tyrosine	2.1
Glycyl-L-phenylalanine (4)	1	Glycyl-L-tyrosine†	1.3
L-Glutamyl-L-phenylalanine†	3	L-Glutamyl-L-tyrosine (5)	1.9
		L-Leucyl-L-tyrosine‡	1.4
		L-Tyrosyl-L-tyrosine (5)	0.7
		L-Tyrosylglycine†	1.3
L-Phenylalanylglycine (4)	1.5		
L-Phenylalanylglycinamide acetate (6)	4		
L-Phenylalaninamide acetate (6)	33	L-Tyrosinamide acetate (7)	31
Glycyl-L-phenylalaninamide acetate (6)	95	Glycyl-L-tyrosinamide acetate (6)	64
Carbobenzoxylglycyl L-phenylalanine (8)	401	Carbobenzoxylglycyl-L-tyrosine (9)	235
Phenylpyruvic acid	9	p-Hydroxyphenylpyruvic acid	110
L-3,4-Dihydroxyphenylalanine	>102	L-3,4-Dihydroxyphenylalanine	14

* The figures in parentheses refer to bibliographic reference numbers.

† The synthesis of this compound is described in the experimental section.

‡ Hoffmann-La Roche preparation

parent amino acid as a growth factor is similar for the phenylalanine and tyrosine series. If it is assumed that the controlling factor in the utilization of the phenylalanine and tyrosine peptides is the rate at which they are hydrolyzed by the bacterial enzymes, then the similarity in the relative effectiveness of the two series is not unexpected in the light of current views concerning the specificity of proteolytic enzymes (10).

Utilization of Acetylphenylalanine and Acetyltyrosine—The similarity

in the behavior of phenylalanine and tyrosine derivatives, noted above, was also observed in the case of the acetyl amino acids. Acetyl-L-phenylalanine and acetyl-L-tyrosine, in high concentrations, were unable to promote the growth of the *phenylalanineless* and *tyrosineless* mutants respectively (Table II).

In contrast to the data obtained with *Escherichia coli*, acetyl-DL-phenylalanine has been found to replace phenylalanine in the diet of the growing rat (16). In addition, recent data (17) have given support to the view (18, 19) that acetyl amino acids are normal intermediates in the amino acid metabolism of animal tissues.

Utilization of Phenylpyruvic and p-Hydroxyphenylpyruvic Acids—Examination of the effectiveness of the keto acids corresponding to phenylalanine and tyrosine for the growth of the appropriate mutants showed that phenylpyruvic acid was utilized by the *phenylalanineless* strain for growth, while high concentrations of *p*-hydroxyphenylpyruvic acid were required to show a slight growth-promoting effect on the *tyrosineless* mutant (Table I).

The simplest interpretation of these results would be that the *phenylalanineless* mutant, and presumably the wild type strain, is able to convert phenylpyruvic acid to phenylalanine by direct amination, while the *tyrosineless* strain, and presumably the wild type strain, performs the analogous reaction in the synthesis of tyrosine at a very slow rate. The appreciable difference in the rate of utilization of phenylpyruvic acid by the *phenylalanineless* mutant and of *p*-hydroxyphenylpyruvic acid by the *tyrosineless* mutant suggests that the metabolism of the two keto acids may follow different pathways in *Escherichia coli*. A similar conclusion has been reached by Scalock *et al.* (20) in a study of the metabolism of these keto acids in the scorbutic guinea pig.

Although phenylpyruvic acid is active as a growth factor for the *phenylalanineless* strain, this mutant cannot utilize phenylpyruvylglycine in place of phenylalanine (Table II). The hydrolysis of the keto acylamino acid to form phenylpyruvic acid, therefore, does not proceed at a rate sufficient to promote growth. In addition, the possible amination of the keto acylamino acid to yield phenylalanylglycine, which is an active growth substance, also does not occur at a rate sufficient to support growth. Keto acylamino acids have been suggested as possible intermediates in biological peptide synthesis (21, 22). The *phenylalanineless* mutant, however, apparently does not synthesize phenylalanylglycine from the corresponding phenylpyruvylamino acid.

Effect of Peptides of Dehydrophenylalanine and Dehydrotyrosine—There has been some discussion as to the possible rôle of derivatives of aminoacrylic acid (dehydroalanine) in the intermediary metabolism of amino acids and peptides (23, 24). This question could be explored with the aid of the *phenylalanineless* and *tyrosineless* mutants, and experiments were

performed in which several derivatives of α -aminocinnamic acid (dehydrophenylalanine) or α -amino-*p*-coumaric acid (dehydrotyrosine) were tested for their ability to replace the parent amino acids.

TABLE II

Effect of Phenylalanine and Tyrosine Derivatives on Growth of Mutant Strains of Escherichia coli

The figures in brackets denote the maximal amount of compounds, in $\text{mm} \times 10^4$ per 10 ml., used in the tests.

Compound (figures in parentheses, bibliographic reference Nos.)	Phenylalanineless strain		Tyrosineless strain	
	Activity in place of phenylalanine	Sparing action on phenylalanine requirement	Activity in place of tyrosine	Sparing action on tyrosine requirement
L-Phenylalanine	+		— [212]	— [212]
D-Phenylalanine	— [121]			
DL-Phenylalanine	+		— [363]	— [363]
Glycyl-L-phenylalanine	+		— [90]	— [90]
Glycyl-D-phenylalanine*	— [90]			
Glycyldehydrophenylalanine (11)	— [91]	— [46]	— [460]	— [276]
Glycyldehydrophenylalanylglycine† (11)	— [360]	— [216]	— [360]	— [216]
Glycyldehydrophenylalanylglutamic acid ammonium salt (11)	— [55]			
Glycylphenylserine (12)	— [84]	— [42]		
Acetyl-L-phenylalanine	— [100]	— [50]	— [100]	
Acetyl-DL-phenylalanine	— [100]			
Acetyldehydrophenylalanine (13)	— [100]	— [50]		
Phenylpyruvic acid†	+		— [61]	— [61]
Phenylpyruvylglycine (14)	— [100]			
Phenylpyruvylglycineoxime (14)	— [85]	— [43]		
L-Tyrosine	+ at [55–100]‡	+ at [110]‡	+	
DL-Tyrosine	— [110]	— [110]	+	
Glycyl-L-tyrosine	— [83]	— [42]	+	
Acetyl-L-tyrosine	— [90]		— [134]	— [45]
Acetyldehydrotyrosine (15)			— [270]	— [270]
<i>p</i> -Hydroxyphenylpyruvic acid†	— [55]	— [55]	+	
Phenol			— [11]	— [11]
Phenol + DL-serine§			—	—
1,3,4-Dihydroxyphenylalanine¶¶	— [102]	— [51]	+	

* This compound, when tested in quantities up to 90×10^{-4} mm per 10 ml., did not inhibit the growth of the *phenylalanineless* strain in the presence of glycyl-L-phenylalanine. The synthesis of this compound is described in the experimental section.

† Compound tested by adding filter-sterilized solution to autoclaved media.

‡ Activity ascribed to traces of phenylalanine present as impurity.

§ Phenol used in amounts of 6 and 11×10^{-4} mm per 10 ml., and tested in the presence of 1 to 5 molar equivalents of DL-serine.

¶ Compound tested in the absence and presence of ascorbic acid, as described in the text.

Although glycyl-L-phenylalanine was, on a molar basis, approximately as active a growth factor as L-phenylalanine, glycyldehydrophenylalanine as well as glycyldehydrophenylalanylglycine and glycyldehydrophenylalanylgutamic acid were inactive (Table II). The *phenylalanineless* strain of *Escherichia coli* appears, therefore, to be unable to hydrogenate the α - β double bond of the dehydropeptides to yield the corresponding saturated peptides. Furthermore, the mutant is incapable of hydrolyzing glycyldehydrophenylalanine to glycine, ammonia, and phenylpyruvic acid, since the last named compound, if formed, would have acted as a growth factor in place of phenylalanine. The existence of an enzyme which specifically hydrolyzes dipeptides containing dehydroamino acids has been demonstrated in extracts of animal tissues (25), but does not appear to be present in an active form in the bacterial cultures used in this study.

It is possible that the bacteria can actually perform either the reduction or the hydrolysis of glycyldehydrophenylalanine but at a rate not sufficiently rapid to produce bacterial growth under the experimental conditions employed in this study. However, since none of the dehydropeptides tested showed any activity even in the presence of suboptimal amounts of phenylalanine, i.e. they did not exert a sparing action on the phenylalanine requirement of the mutant, it is doubtful whether they can serve as normal intermediates in phenylalanine synthesis by the mutant strain. It is also doubtful whether the dehydropeptides serve as normal intermediates in phenylalanine synthesis in the wild type strain of *Escherichia coli*, since the anabolism of this amino acid in the mutant and wild type strains should be identical, except for a single gene-controlled reaction which is lacking in the mutant (3). This gene-controlled reaction, in the case of the *phenylalanineless* mutant, is probably involved in the synthesis of phenylpyruvic acid, since the mutant is able to convert phenylpyruvic acid to phenylalanine. Unless dehydrophenylalanine peptides are the only precursors of phenylpyruvic acid, which appears unlikely, the gene-controlled reaction which is lacking in the mutant cannot involve the conversion of such peptides to phenylalanine.

Not only are the peptides containing dehydrophenylalanine inactive in promoting the growth of the *phenylalanineless* mutant, but acetyldehydrophenylalanine is similarly inactive. Furthermore, the *tyrosineless* strain is unable to grow when supplied with acetyldehydrotyrosine. It will be recalled that acetyl-L-phenylalanine and acetyl-L-tyrosine do not serve as growth factors for these mutants. Thus, reduction of the dehydro compounds to the corresponding acetylamino acids would not be detected by the experimental technique employed in this study.

Consideration was given to the possibility that tyrosine may be synthesized from a precursor other than the keto acid. Since tryptophan

has been shown to be formed from indole and serine by *Neurospora* (26), an analogous mechanism for the synthesis of tyrosine from phenol and serine was tested with the *tyrosineless* strain of *Escherichia coli*. No indication of tyrosine synthesis was obtained, however, although the concentrations of phenol employed were without any toxic effect on the growth of the mutant in the presence of tyrosine (Table II).

Utilization of L-3,4-Dihydroxyphenylalanine—The possible activity of L-3,4-dihydroxyphenylalanine (dopa) as a growth factor for both mutant strains also was studied. Since test solutions of minimal medium supplemented with dopa darkened markedly during the 24 hour incubation period, it was necessary to correct the density measurements made with the Evelyn colorimeter for the color developed. Other growth tests were carried out in the presence of ascorbic acid (5 mg. per 10 ml. of medium) which completely prevented oxidation of dopa but did not inhibit or accelerate the growth of the mutants in the presence of phenylalanine or tyrosine. The dopa used in these experiments was a Hoffmann-La Roche preparation.

Dopa was found to be completely inactive as a growth factor for the *phenylalanineless* strain (Table II). Nor did it show any sparing action on the phenylalanine requirement of this strain. However, it had appreciable activity for the *tyrosineless* mutant. The concentration giving half maximal growth was 14×10^{-4} mm per 10 ml. (Table I). The same value was obtained in tests carried out in the absence and in the presence of ascorbic acid.

To our knowledge, this is the first instance in which dopa has been found to serve in place of tyrosine in an *in vivo* system.

Interrelationships between Tyrosine and Phenylalanine Metabolism in Escherichia coli—From the experiments with the *phenylalanineless* and *tyrosineless* mutant strains of *Escherichia coli* some information pertaining to the metabolic relationships between phenylalanine and tyrosine in this organism also has been obtained. In contrast to the data for the rat, which can perform the *in vivo* conversion of dietary phenylalanine to tyrosine (27), phenylalanine does not seem to serve as a precursor of tyrosine for the *Escherichia coli* mutants. As mentioned earlier, the *phenylalanineless* mutant will not grow in a medium containing tyrosine or its derivatives in place of phenylalanine. Nor does tyrosine, or any of the compounds which serve as a source of tyrosine for the *tyrosineless* mutant, exert any detectable sparing action on the phenylalanine requirement. Such a sparing action would certainly be expected if part of the phenylalanine required by the *phenylalanineless* mutant served as an *in vivo* source of tyrosine. It is of interest also that the *tyrosineless* mutant strain could not utilize phenylalanine or phenylalanine derivatives as a source of tyrosine.

Additional evidence in support of the view that phenylalanine is not converted into tyrosine by the *phenylalanineless* mutant was obtained in two large scale experiments in which the mutant strain was grown in the presence of 75 mg. of L-phenylalanine, and acid hydrolysates of the bacteria were assayed for phenylalanine and for tyrosine. The mutant strains were found to be satisfactory assay organisms for these two amino acids in recovery experiments with the bacterial hydrolysates to which known quantities of phenylalanine or tyrosine had been added.

In one large scale experiment, the hydrolysate was found to contain about 60 mg. of L-phenylalanine and, in another experiment, about 80 mg., i.e., approximately as much phenylalanine as had been added to the medium in which the bacteria were grown. Since the hydrolysate in the first experiment contained about 35 mg. of L-tyrosine and 49 mg. in the second experiment, the sum of the bacterial phenylalanine and tyrosine was very much greater than that which could result if the exogenous phenylalanine served as a source of tyrosine as well as of phenylalanine.

These data are difficult to reconcile with the recent conclusion (28), drawn from inhibition studies with β -2-thienylalanine, that in *Escherichia coli* phenylalanine is converted to tyrosine by direct oxidation.

EXPERIMENTAL

Production of Mutant Strains—The production and isolation of strain 58-278 by x-ray treatment of strain 58 has been described previously (2). Strains 58-4899, 58-5030, and 58-6314 were obtained following the treatment of strain 58 with methyl-bis (β -chloroethyl)amine. Strains 58-4899 and 58-5030 were isolated from a culture of strain 58, which was exposed for 20 minutes to a 0.1 per cent solution of the amine and then incubated for 4 hours prior to plating out on complete medium (3). Strain 58-6314 was isolated from a culture of strain 58 exposed for 30 minutes to a 0.1 per cent solution of the amine in citrate buffer of pH 6.25 and incubated for 5 hours prior to plating out on complete medium (3).

Testing of Compounds—For the determination of the activity of phenylalanine or tyrosine derivatives as growth factors for the mutant strains, the organisms were inoculated into 10 ml. of minimal medium (1) containing 0.01 γ of biotin and graded amounts of the test compound. The results of such tests were recorded after incubation of the culture tubes at 30° for 24 hours.

All test compounds were added to the minimal medium prior to sterilization of the latter by autoclaving. In some cases, as noted in Table II, a filter-sterilized solution of the test compound was prepared and added, with sterile technique, to the autoclaved minimal medium in order to avoid possible decomposition of the compound due to autoclaving. In only one instance was there any difference between the results obtained by the two

methods. While filter-sterilized solutions of glycyldehydrophenylalanyl-glycine showed no activity, autoclaved solutions of this substance exhibited a slight activity for the *phenylalanineless* strain (Table II). This may be attributed to slight decomposition of the peptide to yield phenylpyruvic acid.

For the measurement of the rate of growth, the density of the cultures was measured by means of an Evelyn photoelectric colorimeter with filter No. 540. The concentration of each compound which produced half maximal growth was estimated from a growth curve. The relative activity of a test compound as compared to L-phenylalanine or L-tyrosine was calculated for the test and reference compounds from data obtained on the same day.

In order to study the possible sparing action of test compounds, these were added to a medium containing sufficient L-phenylalanine (1×10^{-4} mm per 10 ml.) or L-tyrosine (0.9×10^{-4} mm per 10 ml.) to permit half maximal growth.

L-Glutamyl-L-phenylalanine—2 gm. of the carbobenzoxydipeptide (29) were hydrogenated in methanol in the presence of palladium black. The peptide separated out during the hydrogenation and was dissolved by the addition of water. The filtrate from the catalyst was evaporated and, on addition of alcohol, the peptide crystallized. Yield 1.1 gm. The substance was dried at 78° *in vacuo* over phosphorus pentoxide

$C_{14}H_{18}O_5N_2$.	Calculated.	C 57.0, H 6.2, N 9.5
294.3	Found.	" 56.8, " 6.3, " 9.5

$[\alpha]_D^{25} = +27.0^\circ$ (2% in water + 1 equivalent of HCl)

Glycyl-L-tyrosine—3 gm. of the carbobenzoxydipeptide (9) were hydrogenated in the usual manner. Yield 1.7 gm. The substance was dried at 78° *in vacuo* over phosphorus pentoxide.

$C_{11}H_{14}O_4N_2$.	Calculated.	C 55.5, H 5.9, N 11.75
238.2	Found.	" 55.4, " 5.9, " 11.5

$[\alpha]_D^{25} = +43.7^\circ$ (2% in water + 1 equivalent of HCl)

L-Tyrosylglycine—2.5 gm. of the carbobenzoxydipeptide (9) were hydrogenated in the usual manner. Yield 1.5 gm. The substance was dried at 78° *in vacuo* over phosphorus pentoxide.

$C_{11}H_{14}O_4N_2$.	Calculated.	C 55.5, H 5.9, N 11.75
238.2	Found.	" 55.6, " 5.7, " 11.8

$[\alpha]_D^{25} = +65.9^\circ$ (2% in water + 1 equivalent of HCl)

Glycyl-D-phenylalanine—This compound was prepared in the same manner (4) as was the L form.

$C_{11}H_{14}O_4N_2$.	Calculated.	C 59.4, H 6.3, N 12.6
222.2	Found.	" 59.5, " 6.2, " 12.7

$[\alpha]_D^{25} = -41.7^\circ$ (2% in water)

SUMMARY

The utilization of derivatives of phenylalanine and tyrosine has been studied with artificially produced mutant strains of *Escherichia coli* which require for growth exogenous sources of L-phenylalanine and L-tyrosine. Simple peptides of phenylalanine and tyrosine were found to serve as growth factors for the *phenylalanineless* and *tyrosineless* mutants respectively. Phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid also were capable of replacing phenylalanine and tyrosine. However, acetylphenylalanine and acetyltyrosine were inactive in growth tests.

The relative efficacy of a given peptide as a growth factor was similar for the phenylalanine and tyrosine series, but phenylpyruvic acid showed a relatively greater activity for the *phenylalanineless* mutant than *p*-hydroxyphenylpyruvic acid showed for the *tyrosineless* mutant.

The *phenylalanineless* strain was unable to use peptides of dehydrophenylalanine for growth. Likewise, acetyldehydrophenylalanine or acetyldehydrotyrosine did not serve as growth factors for the *phenylalanineless* or *tyrosineless* mutants.

Data are presented to show that the mutant strains do not synthesize tyrosine from phenylalanine.

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A MICROCOLORIMETRIC METHOD FOR THE DETERMINATION OF CITRIC ACID*

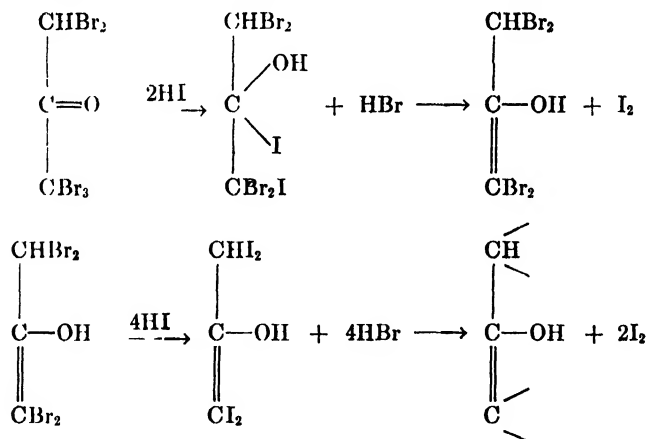
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Kometiani, in 1931 (1), developed a titrimetric method for the macro-determination of citric acid (4 to 40 mg.) based on the reaction of warm alcoholic solutions of organic bromides with sodium or potassium iodide, which had been described by Finkelstein in 1910 (2). Citric acid was first oxidized and brominated to pentabromoacetone. The pentabromoacetone which precipitated quantitatively was filtered off and dissolved in alcohol, to which acetic acid was added to favor the substitution of bromine by iodine. The alcoholic solution was then heated to just below boiling on a water bath, with the addition of an alcoholic solution of sodium iodide. The iodine liberated was titrated with sodium thiosulfate.

The reaction between pentabromoacetone and sodium iodide in acid solution is believed to take place as shown in the accompanying scheme.



Thus, 1 molecule of citric acid = 6I.

By the substitution of colorimetry for titration, it has been found possible to adapt this procedure, with suitable modifications, to the estima-

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tion of citric acid in blood and urine in amounts as small as 10 γ . In the method to be described, the conversion of citric acid to pentabromoacetone is carried out by a modification of the method of Goldberg and Bernheim (3). The substitution of bromine by iodine and the liberation of a colored iodine complex with alcoholic sodium iodide is then carried out according to the reaction of Finkelstein, as applied to pentabromoacetone by Kometiani. The resultant color is measured by means of the Klett-Summerston photoelectric colorimeter.

EXPERIMENTAL

Reagents—

1. Citric acid stock solution; prepared from potassium citrate, $K_3C_6H_5O_7 \cdot H_2O$, and 1 N sulfuric acid. 1.6894 gm. of potassium citrate are dissolved and diluted to 1 liter with 1 N sulfuric acid. This solution is stable for at least a year. 1 cc. contains 1 mg. of anhydrous citric acid; lower concentrations are freshly prepared by appropriate dilutions of the stock solution.

2. Trichloroacetic acid, 10 per cent. 100 gm. of trichloroacetic acid are dissolved and diluted to 1 liter with distilled water.

3. Trichloroacetic acid, 9.3 per cent; 93 gm. of trichloroacetic acid per liter.

4. Sulfuric acid, 27 N. 750 cc. of concentrated sulfuric acid are slowly added to 250 cc. of distilled water; after cooling, the solution is further diluted to 1 liter.

5. Bromide-bromate solution, 2 N. 42.88 gm. of NaBr and 12.58 gm. of $NaBrO_3$ are dissolved and diluted to 250 cc. with distilled water.

6. Hydrazine solution, 10 N. 65 gm. of $N_2H_4 \cdot H_2SO_4$ are dissolved with the aid of 100 cc. of 20 per cent NaOH and diluted to 200 cc.

7. Potassium permanganate, 5 per cent. 50 gm. of $KMnO_4$ are diluted to 1 liter and boiled for 10 minutes. After cooling to room temperature the solution is filtered through glass wool and adjusted to volume.

8. Manganese sulfate, 40 per cent. 400 gm. of $MnSO_4 \cdot H_2O$ are diluted to 1 liter and heated to about 50° until dissolved; after cooling to room temperature the solution is filtered and adjusted to volume.

9. Normal heptane, b.p. 98°. A pure grade (99 mole per cent pure) is employed which can be used without further purification.¹

10. Alcoholic sodium iodide solution, 10 per cent. The required amount is dissolved and diluted with 95 per cent ethyl alcohol. The solution is made up in amber-colored volumetric flasks to minimize the liberation of iodine by the influence of light. This should be freshly prepared before use.

¹ We are indebted to Mr. Leader of the Phillips Petroleum Company, Bartlesville, Oklahoma, for his helpfulness in obtaining a satisfactory grade of normal heptane.

11. Bromocresol green indicator solution, 0.04 per cent. 400 mg. are dissolved in 6 cc. of NaOH, 0.1 N, and diluted to 1 liter.

Method

The steps in the analytical procedure are identical for blood serum, plasma, and urine except for the use of different dilution factors. Citric acid is converted in a strongly acid solution to pentabromoacetone, which is then extracted with heptane and treated with alcoholic NaI. The intensity of the resultant color is determined in the Klett-Summerson photoelectric colorimeter with Filter 42.

Procedure for Determination of Citric Acid in Serum or Plasma

Preparation of Protein-Free Filtrate—Either serum or oxalated plasma is used. Heparin is an unsuitable anticoagulant, since it interferes with the citric acid determination. 1.5 cc. of serum or plasma are added to 11 cc. of 10 per cent trichloroacetic acid in a 15 cc. centrifuge tube. It was found convenient to have volumetric pipettes made up of these capacities. The mixture is well stirred, allowed to stand at room temperature for about 10 minutes, and then centrifuged for the same period of time at about 1500 R.P.M. The supernatant is filtered through a 4 cm. No. 42 Whatman dry filter paper into a 25 cc. Erlenmeyer flask.

When abnormal amounts of acetone bodies are present in the blood, the filtrate is prepared and treated as follows: 3 cc. of plasma are used instead of 1.5 cc. and added to 22 cc. of 10 per cent trichloroacetic acid; 20 cc. of the protein-free filtrate are pipetted into a 50 cc. Erlenmeyer flask and the volume reduced to about 6 cc. by boiling on a hot-plate. After filtration into a 25 cc. graduated cylinder and readjustment when cool to the original volume of 20 cc., a 10 cc. aliquot is used for analysis. This preliminary treatment removes acetone and acetoacetic acid. β -Hydroxybutyric acid interferes to some extent; the interference is not destroyed by heating but is subsequently reduced through the preliminary bromination.

Preliminary Bromination and Extraction—10 cc. of the protein-free filtrate are pipetted into a 30 cc. glass-stoppered Pyrex bottle to which 5 cc. of 27 N sulfuric acid from a burette and 1 cc. of the bromide-bromate mixture are added under a hood. The mixture is allowed to stand at room temperature for about 30 minutes until it has reached room temperature. 0.2 cc. of hydrazine is added and the mixture shaken until the free bromine is reduced. At this point, the procedure can be interrupted, if desired, and the filtrate kept in the ice box for several days with no loss of citric acid. 6 cc. of heptane are now added and the bottle is stoppered and shaken in a Schaerr shaking machine (or any suitable shaking device) for about 3 minutes. After there has been a complete separation of

the two phases, 15 cc. of the lower aqueous phase are transferred to a 60 cc. glass-stoppered Pyrex bottle and cooled in a water bath maintained at about 18°.

Oxidation of Citric Acid to Pentabromoacetone—1 cc. of manganese sulfate solution, 1 cc. of the bromide-bromate mixture, and 2 cc. of the potassium permanganate solution are now added, and the mixture is well shaken and allowed to stand in the water bath at about 18° for about 30 minutes. Excess bromine and manganese dioxide are then reduced by the dropwise addition of hydrazine until the brown color has completely disappeared.

Extraction of Pentabromoacetone—11 cc. of heptane are added and the mixture is shaken, with the bottle well stoppered, in the Schaerr shaker for 6 minutes. The contents are next poured into a 60 cc. separatory funnel. A strictly quantitative transfer to the separatory funnel is not necessary, since an aliquot is subsequently used. After complete separation of the two phases, the aqueous phase is discarded and the heptane extract washed twice with a jet of water from a wash bottle, to which a few drops of bromocresol green solution have been added. The washing must include the neck and the stopper. About 10 cc. of the distilled water are then added, the funnel is stoppered and shaken for about 20 seconds, and the wash water discarded. This is repeated until the color of the wash water is identical with that of distilled water containing the indicator. Two repetitions usually suffice. The mixture is then allowed to stand for about 30 minutes to insure complete drainage of the wash water to the bottom of the funnel; the aqueous phase is then removed quantitatively. The procedure may also be interrupted at this stage, if desired, since the heptane extract remains stable in the ice box for at least 2 weeks.

Color Development and Estimation—10 cc. of the heptane extract are then pipetted into a small amber-colored glass-stoppered volumetric flask or bottle. To this are added 3 cc. of the alcoholic sodium iodide solution; the mixture is shaken and kept stoppered in the dark for about 70 minutes. The heptane, together with the alcoholic phase, is transferred to a colorimeter tube and the intensity of the color read in the Klett-Summerson photoelectric colorimeter with Filter 42. The volume of the alcoholic phase in which the color develops is 2.8 cc.; hence, micro colorimeter tubes are used or a small plug is placed at the bottom of the slot to permit the use of macro colorimeter tubes. The upper surface of the plug should come just below the shelf on which the micro tube ordinarily rests in order to avoid errors due to the rounded bottom of the macro tube. When this is done, the meniscus of the alcoholic phase will be safely above the upper margin of the slot through which the light is transmitted. This convenient device permits the use of the less expensive and sturdier macro tubes.

Analysis of Standard Solutions—Standard solutions containing 100 and 200 γ of citric acid per 10 cc. are analyzed by the same procedure. These solutions are freshly prepared by appropriate dilutions of the stock solution in the following way. The stock solution is first diluted 1:4. 2 and 4 cc. of the resultant solution are then further diluted to 50 cc. in a volumetric flask with the addition of 45 cc. of 10 per cent trichloroacetic acid.

The analytical procedure for the standard solutions is identical with that for the blood filtrates, except for the amount of the final heptane extract taken for color development. Instead of 10 cc. as with serum or plasma, 2 cc. of the final heptane extract are used. This is made up to 10 cc. with heptane, and 3 cc. of the sodium iodide solution are added. The final concentrations of citric acid thus represent 20 and 40 γ . The remainder of the final heptane extract may be used for subsequent analyses since it is stable for at least 2 weeks.

Calculation of Results

$$\frac{(\text{Reading of unknown}) - (\text{blank (calculated)})}{\text{Reading with 10 } \gamma} \times \frac{100}{12 \text{ (cc. aliquot of plasma)}} = \text{mg } \% \text{ citric acid}$$

The blank is calculated as follows:

$$(\text{Reading with } 20 \gamma) - ((\text{reading with } 40 \gamma) - (\text{reading with } 20 \gamma)) = \text{blank (calculated)}$$

$$\frac{(\text{Reading with } 20 \gamma) - (\text{blank (calculated)})}{20} = \text{reading with } 10 \gamma$$

Procedure for Determination of Citric Acid in Urine

Since the amount of citric acid in urine varies over a wide range, provisions have been made in the procedure to use smaller or larger volumes of urine for analysis.

Normal Urine—The usual range of urinary citric acid values is dealt with by adding 9.5 cc. of 9.3 per cent trichloroacetic acid to 0.5 cc. of urine in a 30 cc. glass-stoppered Pyrex bottle and carrying out the analytical procedure as described for serum or plasma.

Should the urinary citric acid concentration fall below the range of accuracy of the method, then 1 cc. of urine can be used and diluted with 9 cc. of 10 per cent trichloroacetic acid, or 2 cc. of urine + 8 cc. of trichloroacetic acid, 11.2 per cent; etc. In each case, 10 cc. of diluted urine will contain the same amount of trichloroacetic acid.

The procedure for urine involves one further difference from that for blood; namely, the use of 2 cc. instead of 10 cc. of the final heptane extract for color development. Of the final heptane extract, 2 cc. are taken and diluted to 10 cc. with heptane in a small amber-colored glass-stoppered

volumetric flask or bottle prior to the addition of 3 cc. of alcoholic sodium iodide.

The presence in urine of glucose, albumin, or acetone bodies interferes with the accuracy of citric acid determinations. This interference is eliminated in the following manner.

Urine Containing Glucose—1 cc. of urine is diluted to 100 cc. with the addition of 90 cc. of 10 per cent trichloroacetic acid. 10 cc. are then carried through the analytical procedure in the manner described for serum or plasma, with 10 cc. of the final heptane extract for color development.

Urine Containing Albumin—1 cc. of urine is diluted to 20 cc. with 10 per cent trichloroacetic acid, filtered, and 10 cc. of the filtrate used for analysis as in normal urine.

Urine Containing Acetone Bodies—1 cc. of 27 N sulfuric acid is added to 25 cc. of urine, which is then reduced by boiling to about 10 cc., cooled, and made up to 25 cc. in a volumetric flask. 0.5 cc. is then diluted with 9.5 cc. of 9.3 per cent trichloroacetic acid. The analysis is carried out as for normal urine.

Calculation of Results The formula is given for calculating the result of the analysis when either 0.5 or 1.0 cc. of urine is diluted to a final volume of 10 cc., and 2 cc. of the final heptane extract are used for the color reaction.

$$\frac{(\text{Reading of unknown}) - (\text{blank (calculated)})}{\text{Reading with } 1.0 \gamma} \times \begin{array}{l} \text{volume (in cc) per 24 hrs} \\ 100 \text{ (for 0.5 cc urine) or } 200 \text{ (for 1.0 cc urine)} \\ = \text{mg citric acid per 24 hrs} \end{array}$$

The blank and reading with 1.0 γ are calculated as for blood.

DISCUSSION

Stability of Citric Acid in Urine and Blood—Citric acid in urine was found to be stable for a period of at least a week, when 24 hour specimens were preserved with 25 cc. of 18 N sulfuric acid and kept under refrigeration. Citric acid in blood remained constant when whole blood was left in the ice box for 2, 6, and 12 hours; there was a slight increase after 24 hours.

Influence of Acidity—Goldberg and Bernheim, in describing their method for determining 1 to 40 mg. of citric acid, have pointed out the necessity for the preliminary bromination and have elucidated the mechanism of the oxidation-bromination procedure. They likewise observed that, up to a certain maximum, increasing the acidity during oxidation yielded higher recoveries of citric acid. Maximal recoveries occurred in approxi-

mately 8 N sulfuric acid; thereafter, there was a sharp falling off of the values obtained. We have been able to confirm the influence of acidity on the recovery of the smaller amounts of citric acid measured by our method, except for the slope of the curve which fell less abruptly with acidities greater than 8 N. In Fig. 1 are plotted the colorimeter readings for 20 γ of citric acid when varying normalities of acid were used during oxidation. We have accordingly chosen an approximately 8 N solution for the determination of 10 to 60 γ of citric acid.

Oxidation of Citric Acid to Pentabromoacetone—Goldberg and Bernheim have emphasized the importance of the very slow addition of permanganate in the oxidation of citric acid to acetonedicarboxylic acid. In their procedure manganese sulfate was added to hasten the formation of manganese

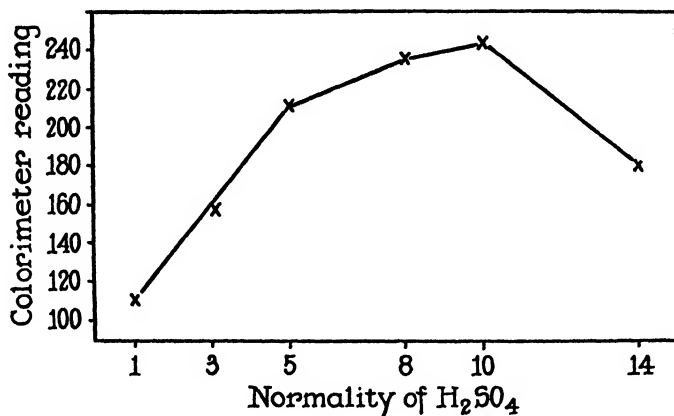


FIG. 1. Color developed with 20 γ of citric acid as a function of the normality of sulfuric acid used before oxidation.

dioxide, which can be present in excess without decomposing acetonedicarboxylic acid. For the determination of citric acid in the presence of glucose, they suggested the substitution of a manganese dioxide suspension for permanganate and described the preparation of such a suspension. We have investigated the relative merits of both the suspension and the permanganate solution for the determination of the smaller amounts of citric acid for which our method was designed, adding both at once, and found negligible differences. The potassium permanganate solution was therefore selected because of its convenience.

Extraction of Pentabromoacetone—These same investigators used petroleum ether with a boiling point between 35–50° for the extraction of pentabromoacetone from the aqueous phase. This solvent has a number of disadvantages. It must be purified with sulfuric acid and redistilled

before use. In warm weather, its vapor pressure is such that the stoppers are frequently blown out during the shaking, with the loss of some of the reaction mixture. We have, in its stead, employed *n*-heptane (99 mole per cent pure) with a boiling point of 98°, which was obtained from the Phillips Petroleum Company. This solvent has a much lower vapor pressure than the petroleum ether with the lower boiling point, and hence presents no difficulties as regards evaporation or blowing out of the stoppers during warm weather. Its solubility in water is one-fourth of that of the lower boiling petroleum ether. It can be used without further purification. Finally, the heptane extracts have been found stable for at least a week when refrigerated.

Quantitative separation of the heptane extract from the aqueous phase is difficult. This difficulty is avoided by extracting with 11 cc. of heptane and taking a 10 cc. aliquot for the color reaction in the procedure for blood plasma. In the procedure for urine, the extraction is carried out with 11 cc. of heptane and an aliquot of 2 cc. is used for the color reaction. The use of this small aliquot in the urine method has a number of advantages. It permits a repetition of the colorimetric determination on the same extract. With the standard solutions, the remainder of the final heptane extract, because of its stability under refrigeration, may be used for the color reaction for a period of at least a week, thus avoiding the necessity of preparing and analyzing fresh standards with each set of determinations.

We wish to emphasize the necessity for washing the heptane extract completely free of acid. While five washings, including two with shaking of the separatory funnel for 20 seconds, usually sufficed to accomplish this, it was found desirable to provide an additional safeguard in the form of an indicator, which is added to the wash water in the separatory funnel. It was necessary to use an indicator which was soluble in water and insoluble in heptane, with a narrow pH range and a sharp change in color at the pH of distilled water. Bromocresol green, with a pH range of 4.2 to 5.4 and a sharp change in color from yellow through green to blue, fulfilled these requirements.

Finally, about $\frac{1}{2}$ hour of standing is usually required for the quantitative separation of the aqueous phase.

Color Development from Pentabromoacetone in Heptane and Alcoholic Sodium Iodide—We have further investigated the reaction between pentabromoacetone and alcoholic NaI as described by Kometiani. This investigator carried out the reaction in a strongly acid solution at just below boiling. We have found that, under our conditions, the reaction proceeds smoothly and rapidly at room temperature and without acidification. About 90 per cent of the color is developed immediately on mixing

the heptane extract with the alcoholic sodium iodide, however, the reaction is complete on standing for about 1 hour. Thereafter there is very slow uniform increase throughout a set of determinations, due to a slow oxidation of sodium iodide to free iodine.

For the determination of the optimal conditions for the development of the color reaction, a stock solution of pentabromoacetone in heptane was prepared by the oxidation and bromination of citric acid. The heptane

TABLE I
Effect of Different Concentrations of Ethyl Alcohol on Color Development

Citric acid	Ethyl alcohol per 100 cc solution	NaI per 100 cc solution	Colorimeter readings (Klett Summerson)
γ	cc	gm.	
40	100	10	174
40	95	10	221
40	90	10	212
40	80	10	166
40	70	10	126

TABLE II
Effect of Different Concentrations of Sodium Iodide in 95 Per Cent Ethyl Alcohol on Color Development

Citric acid	Sodium iodide in 95 per cent ethyl alcohol	Colorimeter readings (Klett Summerson)
γ	gm per 100 cc	
40	5	181
40	10	220
40	15	238
40	20	262

extract was diluted with heptane to give the concentration of pentabromoacetone equivalent to the desired concentration of citric acid per 10 cc. of solvent. 5 cc. of alcoholic NaI were used throughout.

The total color developed with a definite concentration of citric acid was found to depend on (1) the concentration of ethyl alcohol, and (2) the concentration of sodium iodide, as shown in Tables I and II.

A 10 per cent solution of sodium iodide in 95 per cent alcohol was selected as the most convenient concentration of both elements. The advantage of the higher readings obtained with the higher concentrations of sodium iodide was balanced by the difficulty of getting the larger amounts of sodium iodide into solution. In addition the higher concentrations were so near saturation that crystallization in the colorimeter tube could not be

prevented. Potassium and lithium iodide proved unsatisfactory substitutes for sodium iodide.

The next concern was with the final volume of the color phase. When 1 volume of heptane and 3 volumes of 10 per cent sodium iodide in 95 per cent ethyl alcohol are shaken, complete mixing takes place. With smaller volumes of alcoholic sodium iodide, a definite interface is established. These facts are shown in Table III.

The influence of varying the amounts of alcoholic sodium iodide on the colorimetric reading is shown in Fig. 2.

The selection of the exact amount of alcoholic sodium iodide for use in the procedure was further influenced by practical considerations. The

TABLE III
*Miscibility Characteristics of Heptane and 10 Per Cent Sodium Iodide in
95 Per Cent Ethyl Alcohol*

10 cc. of heptane were used throughout the series.

10 per cent sodium iodide in 95 per cent ethyl alcohol	Lower phase	10 per cent sodium iodide in 95 per cent ethyl alcohol	Lower phase
cc.	cc.	cc.	cc.
1	0.6	9	10.8
2	1.6	10	12.2
3	2.8	15	19.0
4	4.0	20	26.0
5	5.4	25	33.0
6	6.8	30	40.0
7	8.1	35	45.0
8	9.5	40	50.0

The bold-faced figures indicate the ratio at which complete mixing first takes place.

higher reading obtained with a lower phase of 1.6 cc. which resulted from the use of 10 cc. of heptane and 2 cc. of alcoholic sodium iodide would have been preferable to the reading obtained with 3 cc. of alcoholic sodium iodide, which gave a lower phase of 2.8 cc. A lower phase of 1.6 cc. would have required the use of the more expensive and less sturdy micro colorimeter tubes. We therefore employed 3 cc. of alcoholic sodium iodide, and utilized the macro colorimeter tube, adapting the instrument in the manner previously described to the use of the macro tube and for the resultant volume of 2.8 cc.

Characteristics of Brown Color Developed—Absorption spectra were made with a Beckman quartz spectrophotometer to obtain more information about the nature of the color which develops when heptane containing pentabromoacetone is allowed to react with alcoholic NaI. The absorption

characteristics were found to be identical with those of free iodine in an alcoholic solution of NaI, as is evident from Table IV.

In accordance with the absorption spectra, both Klett-Summerson Filters 40 and 42 were investigated. Contrary to expectations, higher colorimetric readings and better proportionality were obtained over the

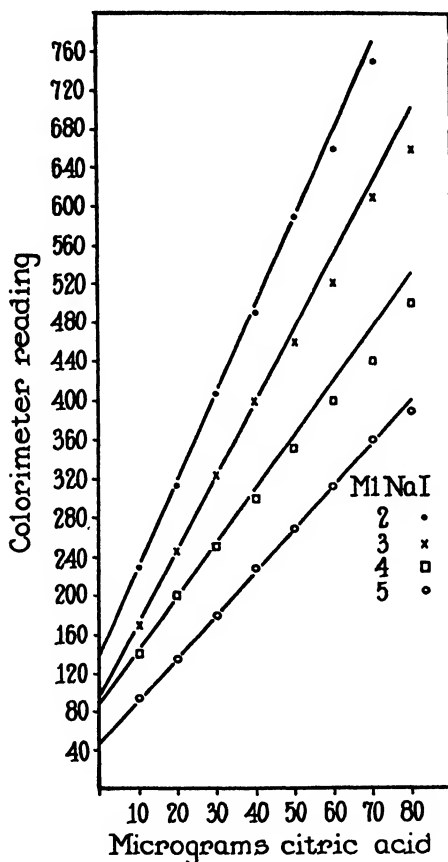


FIG. 2. Total color developed with different concentrations of citric acid and different amounts of 10 per cent sodium iodide in 95 per cent ethyl alcohol.

range of 10 to 60 γ with the latter. This is most likely due to a decreased sensitivity of the instrument at the lower wave-lengths of the visible spectrum. For these reasons Filter 42 was selected. It should be pointed out, in this connection, that not all Filters 42 supplied for this instrument give a uniform performance. Of two such filters on hand, one gave linear proportionality up to a reading of about 400 on the colorimeter, while the

other fell off at about 250, and hence could not be used for the citric acid method.

In the range between 10 and 60 γ of citric acid as determined by this method there is a straight line relationship between the colorimetric reading and the concentration of citric acid.

In Fig. 3 are presented the results obtained over this range of concentrations. Each point represents an arithmetic mean of five determinations at each concentration, the blank value for the reagents having been deducted. These blank values were found to differ with each set of determinations, making it impossible to use a standard calibration curve. Hence, it has been found necessary to run standard solutions for each series of determinations. These fluctuations in the blank values were

TABLE IV

Absorption Characteristics of Reaction Color Complex As Compared with Free Iodine in Alcoholic Solution of Sodium Iodide

The results are expressed in per cent transmission.

Wave length $m\mu$	Reaction color	Free iodine + sodium iodide in ethyl alcohol	Wave length $m\mu$	Reaction color	Free iodine + sodium iodide in ethyl alcohol
320	10	10	410	38	38
330	11	11	420	51	51
340	4	4	430	60	60
350	2	2	450	74	74
360	1	1	480	90	90
370	2	2	520	96	96
380	4	4	540	97	97
390	11	11	600	98	98
400	23	23	650	100	100

apparently not related to small variations in the concentration of the sodium iodide solution, since the use of sodium iodide solutions of definite normality failed to eliminate them. We have not investigated other possible sources of this variation.

The need for the actual determination of the relatively small reagent blank was avoided by running two standards at different concentrations for each series of determinations, as suggested by Barker and Summerson (4). The difference in readings between the two standards provides the basis for the calculation of both the "apparent blank" and the actual concentrations of citric acid.

When readings fall outside the range of accuracy of the urine procedure, it is still possible to salvage the determination. Since there is an excess available of the final heptane extract, the final step, that of color develop-

ment, can be repeated by taking smaller or larger aliquots of the heptane extract, depending on whether the readings are too high or too low, and diluting them to 10 cc. with heptane. With the plasma procedure, if readings are too high, they may be brought within the range of accuracy of the method by diluting the final lower colored phase in both standards and unknown with a definite amount of alcoholic sodium iodide. If readings are too low, the determination must be repeated with larger amounts of plasma.

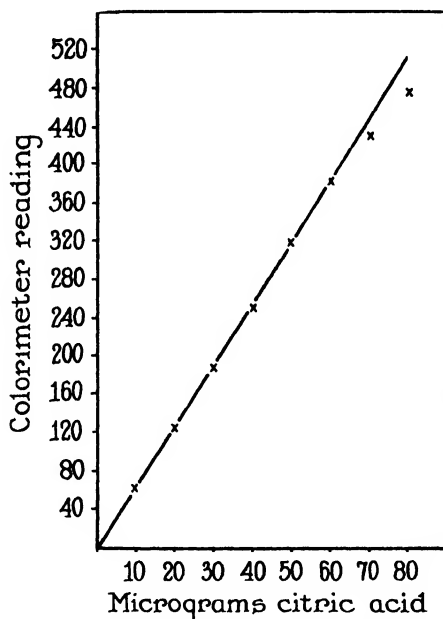


FIG. 3. Color developed as a function of the citric acid concentrations over a range of 10 to 60 γ

Comparison of Colorimetric and Titrimetric Methods; Recovery Experiments—In addition to conventional recovery experiments with urine and blood, a comparison was made of the values for citric acid obtained with this colorimetric method and with the macrotitrimetric method of Goldberg and Bernheim. The values obtained by the microcolorimetric method with a 1.5 cc. aliquot of plasma, and by the macrotitrimetric method with a 40 cc. aliquot are given in Table V.

Similar comparisons were made between the colorimetric method as applied to urine, and the titrimetric method of Goldberg and Bernheim (Table VI).

Recovery experiments were carried out with plasma and urine. With plasma, the desired amount of citric acid was contained in the trichloro-

acetic acid used for the preparation of the protein-free filtrate; with urine, the desired amount of citric acid was added to make the dilution. The results are summarized in Tables VII and VIII.

Determination of Citric Acid in Urine Containing Glucose—The presence of significant amounts of glucose in urine interferes appreciably with the

TABLE V
Comparison of Citric Acid Determinations in Plasma by Microcolorimetric and Macrotitrimetric Methods

Plasma No.	Citric acid		Difference
	Microcolorimetric	Macrotitrimetric	
	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>per cent</i>
1	2.29	2.24	+2
2	2.35	2.55	-8
3	2.13	2.19	-3

TABLE VI
Determination of Citric Acid in Urine by Colorimetric and Titrimetric Procedures

Urine No.	Urine	Citric acid per 24 hrs		Difference
		Colorimetric method	Titrimetric method	
	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1	0.1	293	310	+5
2	0.1	436	438	0
3	0.1	435	443	+2
4	0.1	826	840	+2
5	0.1	465	430	-7
6	0.1	553	560	+1
7	0.5	680	688	+1
8	0.5	908	872	-4
9	0.5	705	720	+2
10	0.5	675	655	-3
11	0.5	215	230	+7
12	0.5	530	505	-5
13	0.5	560	575	+3
14	0.5	848	850	0

accuracy of citric acid determinations. As little as 15 gm. per liter reduces the recovery of citric acid by as much as 30 per cent when it is measured by the titrimetric method of Goldberg and Bernheim. However, when such urines are analyzed, after appropriate dilution, by our colorimetric method, excellent recoveries are obtained in the presence of as much as 100 gm. of glucose per liter. The extent to which glucose may be present

in urine without interference with the recovery of citric acid is shown in Table IX.

TABLE VII
Recovery of Citric Acid Added to Blood Plasma

Plasma No.	Citric acid added	Citric acid found	Citric acid recovered
	γ	γ	<i>per cent</i>
1	0	35.4	104
	20	56.2	
2	0	27.7	96
	20	46.9	
3	0	27.3	101
	20	47.6	
4	0	21.2	102
	15	36.5	

TABLE VIII
Recovery of Citric Acid Added to Urine

Urine No.	Citric acid added	Citric acid found	Citric acid recovered
	γ	γ	<i>per cent</i>
1	0	48.6	98
	20	68.2	
2	0	35.6	92
	20	54.0	
3	0	17.8	100
	10	27.8	
4	0	15.4	104
	10	25.8	

TABLE IX
Determination of Citric Acid in Urine in Presence of Glucose

Urine No. (0.1 cc. samples)	Glucose added to urine	Citric acid found in urine
	<i>gm per l.</i>	<i>mg. per l.</i>
1	0	293
1	20	293
1	50	298
1	100	293
2	0	680
2	50	680
2	100	685

Other Possible Interfering Substances—A large number of substances have been investigated by other workers (5, 6) for their possible interference with the determination of citric acid by its conversion to penta-

bromoacetone. A number were reinvestigated by our procedure and were found not to increase the colorimeter reading when added to 20 γ of citric acid in amounts of 1 mg.; these were pyruvic, succinic, lactic, hippuric, fumaric, malonic, glutaric, α -ketoglutaric, malic, uric, tartaric, oxalic, and ascorbic acids, urea, glucose, thymol, toluene, creatine, and creatinine.

Interference by Acetone Bodies.—Acetone and acetoacetic acids are eliminated, when present in greater than normal amounts, by the procedures described above. The amounts present in normal blood do not interfere. β -Hydroxybutyric acid is, however, not destroyed by boiling in acid solution, but is slowly though not completely eliminated by the preliminary bromination

SUMMARY

1. A microcolorimetric method has been described for the determination of citric acid in small samples of blood and urine.

2. The method is based on the conversion of citric acid to pentabromoacetone and on the subsequent reaction between pentabromoacetone and sodium iodide.

3. This reaction results in the development of a yellow color complex.

4. The range of sensitivity of the method is from 10 to 60 γ .

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PEPTIDE BOND SYNTHESIS

II. THE FORMATION OF *p*-AMINOHIPPURIC ACID BY LIVER HOMOGENATES*

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The demonstration of the enzymatic formation of *p*-aminohippuric acid (PAH) from *p*-aminobenzoic acid (PAB) and glycine (1) has made available a convenient model system for the study of peptide bond synthesis. Because of the complex metabolic relationships existent in surviving tissue and such considerations as diffusion and permeability, the tissue slice technique employed in the preliminary study is of limited value in studying the mechanism and kinetics of the reaction. For this reason, we have turned to the homogenate technique, since it provides a relatively uniform tissue material dispersed to the point where many endogenous reactions are suppressed.

The present study is concerned with the conditions that must be maintained for support of the synthesis by homogenates and provides insight into the requisite source of energy.

Procedures and Methods

Analytical—PAH and PAB were determined by the method described previously (1), in which the proteins are precipitated with trichloroacetic acid, the acid neutralized with sodium hydroxide, the neutral filtrate brought to pH 3.95 with a citrate-phosphate buffer, and the PAB extracted with ether and benzene. Since the medium used for homogenate studies was more highly buffered and the amount of protein in the reaction mixture was high and variable, it was not possible to adjust the alkali concentration so that all samples could be neutralized with a fixed volume. The resultant variation was reflected somewhat in the pH of the buffered extraction mixture, causing differences in the PAH values determined. To avoid the individual titration of each sample, the concentration of the citrate-phosphate buffer was increased 5-fold. The buffer was made by the addition of 0.5 M citric acid to 1.0 M disodium phosphate until the pH upon 3-fold dilution was 3.85. At this new concentration and pH, 1.0 per cent of the PAB and 88 per cent of the PAH remained after extraction, so that

$$\text{PAH} = \frac{(\text{Total after extraction}) - 0.01 (\text{total before extraction})}{0.87}$$

* Aided in part by a grant from the Wisconsin Alumni Research Foundation.

Tissue—Rat liver homogenates were used throughout this study. After the animal was stunned and exsanguinated, the liver was removed and placed on ice. Homogenization was carried out in the cold room, with a glass pestle and with cold isotonic KCl as the suspending medium. The pestle was driven at 3000 to 4000 R.P.M., and the homogenization was continued at least 1 minute after the tissue had been broken up, with rapid movement of the tube up and down the pestle. The homogenate was filtered through glass wool before use.

These conditions insured practically complete disruption of cells. The necessity of an intracellular type of medium and a pronounced dilution effect provide additional evidence for the essentially cell-free nature of the homogenate.

Substrates Coenzyme I (DPN) was prepared from bakers' yeast by the method of Williamson and Green (2) and purified by a new method of LePage (3). The preparation contained 52 per cent of DPN, as determined by the spectrophotometric method. We are indebted to Dr. V. R. Potter for performing the analysis. Material prepared by the method of Williamson and Green was contaminated with heavy metals together with a substance that reduced cytochrome *c* (probably glutathione), and assayed only 20 to 30 per cent of DPN. According to LePage,¹ the chief contaminant of the purified material was adenylic acid. The product did not reduce cytochrome *c*.

Adenosine triphosphate (ATP) was made from rabbit muscle and purified by reprecipitation as the barium salt (4). For use, the salt was dissolved in 0.1 M phosphoric acid, the barium precipitated as the sulfate, and the solution neutralized with KOH.

Cytochrome *c* was prepared from beef hearts by the method of Keilin and Hartree (5) and stored in solution.

PAB was purified as before (1). Fumaric acid was a commercial product recrystallized from boiling HCl to remove traces of maleic acid or anhydride. Glycine, malic acid, L-glutamic acid, citric acid, oxalic acid, aceturic acid, and thiamine pyrophosphate were commercial products. Pyruvic acid was a redistilled commercial preparation. α -Ketoglutaric acid and oxalacetic acid were synthetic products (4). Succinic acid was prepared from the anhydride. Commercial calcium hexose diphosphate² was treated with excess oxalic acid to precipitate calcium, and the calcium-free solution used. The inorganic salts were commercial products selected for low calcium content.

¹ We wish to express our appreciation to Dr. G. A. LePage for his many helpful suggestions concerning the preparation of DPN.

² We are indebted to the Endo Products, Inc., for a generous supply of this compound.

All substrates and media were made up with twice distilled water and brought to the proper pH (usually 7.55) with KOH under the glass electrode. The media were made isotonic with KCl in a final volume of 4.0 ml. per flask; details of the composition are reported with each experiment.

Procedure—The homogenate was immediately added to the cold media in Warburg flasks, and the flasks were kept on ice until placed on the manometers. Alkali was placed in the center wells of the flasks and the gas phase was 100 per cent oxygen, unless otherwise noted. The incubations were carried out with shaking at 38°. Warburg flasks were used for convenience only, since gas exchange was not measured.

The incubations were terminated by placing the flasks in ice water, and an aliquot for analysis was quickly removed and added to previously measured trichloroacetic acid solution.

Our results are reported on the basis of the weight of PAB converted to PAH per mg. of homogenate nitrogen, which was determined on an aliquot of the homogenate by the micro-Kjeldahl method. This method of expressing the results reflects the activity of the homogenate more accurately than does one based on the total weight of the liver used, since this quantity depends on the variable factors of fat, water, and glycogen content which have no relation to the enzymatic activity.

Results

Inorganic Ions—In the initial experiments, adenosine triphosphate (ATP), coenzyme I (DPN), and cytochrome *c* were added, although no requirement for them had yet been shown. Replacement of sodium ion with potassium ion markedly stimulated the reaction (Fig. 1). The shape of the curve indicates that the reaction involves a stimulation by the potassium ion rather than an inhibition by sodium ion. Since the two ions are ordinarily equivalent in reactivity, the effect may be due to an influence on the state of aggregation of the liberated intracellular proteins. In subsequent experiments, potassium salts were employed exclusively.

Calcium ions inhibited the reaction (Fig. 2). This was expected, since the synthesis requires the support of an oxidative metabolism, and calcium ion lowers the respiration of homogenates in general and is an activator for the breakdown of the pyridine nucleotides and adenylyl phosphates (6, 7).

Magnesium ion in low concentration slightly stimulated the formation of PAH (Fig. 3). The maximum stimulation varied with the composition of the experimental medium. Fig. 3 illustrates the effect for low potassium concentrations. At higher potassium concentrations, the amount of stimulation is less, and the optimum concentration range broader. In later experiments when fumarate was added, the stimulation was a maximum of 8 per cent at 0.00038 M magnesium. Although the magnitude of stimu-

lation was slight, magnesium ion was always added in order to maintain maximum activity. It probably exerts its effect secondarily through the

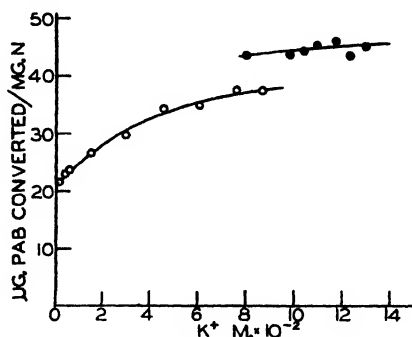


FIG. 1. *p*-Aminobenzoic acid (PAB) converted to *p*-aminohippuric acid per mg. of homogenate nitrogen in two experiments with increasing potassium ion concentration. 0.5 ml., containing 7.5 to 9.5 mg. of nitrogen, of a rat liver homogenate was added to each flask. Final concentrations, 0.001 M PAB, 0.01 M glycine, 0.01 M phosphate at pH 7.40, 0.00096 M magnesium sulfate, 0.000085 M cytochrome *c*, 0.00038 M coenzyme I, 0.0015 M adenosine triphosphate. Total volume of 4.0 ml. made isotonic with NaCl; potassium added as KCl; incubated 60 minutes at 38° under 100 per cent oxygen.

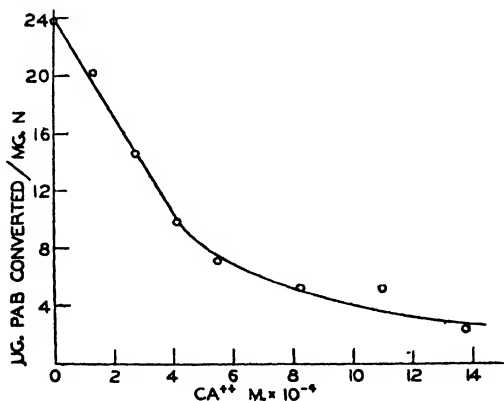


FIG. 2. Inhibitory action of calcium ions on the conversion of *p* aminobenzoic acid (PAB) to *p*-aminohippuric acid. 1.0 ml., containing 9.9 mg. of nitrogen, of a rat liver homogenate was added to each flask. Final concentrations, 0.001 M PAB, 0.01 M glycine, 0.01 M phosphate at pH 7.40, 0.000085 M cytochrome *c*, 0.00038 M coenzyme I, 0.0015 M adenosine triphosphate, 0.00154 M magnesium sulfate, 0.0062 M potassium chloride. Total volume of 4.0 ml. made isotonic with NaCl; incubated 60 minutes at 38° under 100 per cent oxygen.

maintenance of a reaction contributing to the energy required for the synthesis of the peptide bond.

Fluoride (Fig. 4) slightly stimulated the reaction at a low concentration, and inhibited at higher concentrations in the presence of ATP. However, the inhibitory concentrations are low enough to suggest that a transphosphorylation mechanism may be involved in the synthesis.

Phosphate ion was without significant effect on the reaction over the range from 0.01 to 0.06 M (Table I). The slight stimulation observed at higher phosphate concentrations can be accounted for by better buffering action.

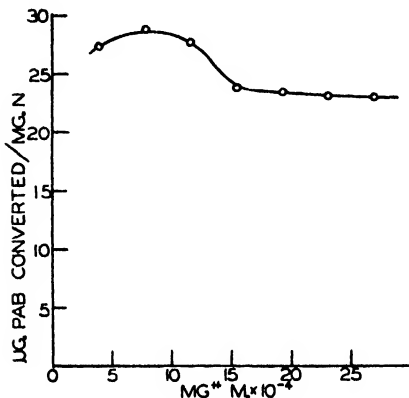


FIG. 3.

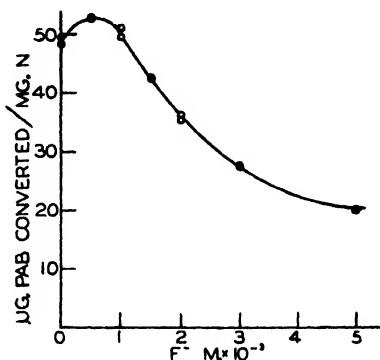


FIG. 4

FIG. 3. *p*-Aminobenzoic acid (PAB) converted to *p*-aminohippuric acid per mg. of homogenate nitrogen with increasing magnesium concentrations. 1.0 ml., containing 9.9 mg. of nitrogen, of a rat liver homogenate added to each flask. Final concentrations, 0.001 M PAB, 0.01 M glycine, 0.01 M phosphate at pH 7.40, 0.0000085 M cytochrome *c*, 0.00038 M coenzyme I, 0.0015 M adenosine triphosphate, 0.0062 M potassium chloride. Magnesium added as the sulfate. Total volume of 4.0 ml. made isotonic with NaCl; incubated 60 minutes at 38° under 100 per cent oxygen.

FIG. 4 The action of fluoride on the aerobic conversion of *p*-aminobenzoic acid (PAB) to *p*-aminohippuric acid. 1.0 ml., containing 6.0 mg. of nitrogen, of a rat liver homogenate was added to each flask. Final concentrations, 0.001 M PAB, 0.01 M glycine, 0.00078 M magnesium sulfate, 0.037 M potassium phosphate at pH 7.55, 0.000012 M cytochrome *c*. Fluoride added as sodium salt; total volume of 4.0 ml. made isotonic with KCl; incubated 45 minutes at 38° under 100 per cent oxygen.

Effect of pH—The synthetic activity of the homogenates was critically dependent on the pH (Fig. 5). We had observed that 0.01 M phosphate buffer, the concentration commonly employed in homogenate studies, was not adequate to maintain the pH at a constant level, particularly in higher tissue concentrations. Consequently, we raised the phosphate concentration 6-fold in order to study the effect of pH, since changes in phosphate concentration had a negligible effect on the rate of the reaction over this range. Even at this higher concentration, the pH dropped from 0.05

TABLE I

Effect of Phosphate on p-Aminohippuric Acid Formation

1.0 ml., containing 7.1 mg. of nitrogen, of a rat liver homogenate was added per flask. Final concentrations, 0.001 M *p*-aminobenzoic acid (PAB), 0.01 M glycine, 0.00078 M magnesium sulfate. Phosphate was added as potassium salt at pH 7.40. Total volume of 4.0 ml. made isotonic with KCl. Incubated 45 minutes at 38° under 100 per cent oxygen.

Phosphate concentration	PAB converted
$M \times 10^{-2}$	γ per mg. N
1.03	27.2
1.54	26.6
2.06	25.9
2.58	26.6
3.09	27.2
3.60	27.6
4.12	28.4
4.64	29.3
5.15	29.8
5.66	29.8
6.18	30.8

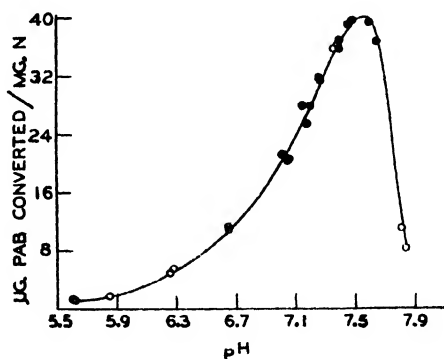


FIG. 5. The effect of varying hydrogen ion concentrations on the production of *p*-aminohippuric acid. Final concentrations, 0.001 M *p*-aminobenzoic acid (PAB), 0.01 M glycine, 0.00078 M magnesium sulfate, 0.062 M potassium phosphate. Total medium adjusted to various pH values before the addition of the homogenate. pH values shown are those of the media at the end of the incubation. Two experiments are shown: the homogenate nitrogen value of the first experiment (O) not known, and the value assumed, so that pH 7.40 values in both experiments coincide; nitrogen value of the second experiment (●) 5.4 mg. per flask. Total volume of 4.0 ml. made isotonic with KCl; incubated 45 minutes under 100 per cent oxygen.

to 0.15 unit in the range of pH 7.2 to 7.6 during the course of the incubation. The results in Fig. 5 are based on the pH (at 25°) of the medium at the end of the incubation time, showing a maximum at pH 7.55. The

initial pH of the buffer for this final value was 7.60 to 7.65. Since the pH curve is steep on the alkaline side of the optimum, we subsequently employed pH 7.55 as the *initial* value for our media in order to provide a slight margin of safety in the event of small errors in adjusting the glass electrode.

The problem of pH control has not been adequately dealt with in many studies employing the homogenate technique, particularly since the sensitivity of the enzyme systems to changes in the hydrogen ion concentration is usually unknown, and the homogenate, unlike intact tissue slices, has little inherent protection against environmental changes.

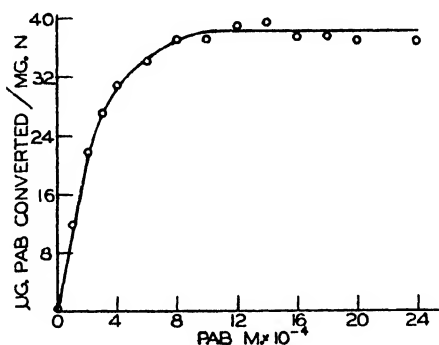


FIG. 6. *p*-Aminohippuric acid production from varying concentrations of *p* amino benzoic acid (PAB). 1.0 ml., containing 4.0 mg. of nitrogen, of a rat liver homogenate was added per flask. Final concentrations, 0.01 M glycine, 0.00078 M magnesium sulfate, 0.058 M potassium phosphate at pH 7.55. Total volume of 10 ml made isotonic with KCl; incubated 45 minutes at 38° under 100 per cent oxygen.

0.01 M phosphate buffer is inadequate to protect a pH-sensitive homogenate system, since an addition of 0.0007 N acid or base will shift the pH 0.2 unit in the region of pH 7.5. Even with higher phosphate concentrations, it is necessary to employ the glass electrode in making up the media, because the substrates are often buffers in their own right, and the practice of adjusting the pH of their solutions with a spot plate is subject to gross error.

Substrate Concentrations—0.001 M PAB was adequate to saturate the system in the presence of 0.01 M glycine (Fig. 6). Variation in the PAB concentration necessitated the use of different dilutions in performing the analysis, thus introducing variable errors which are reflected in the spread of the experimental points in Fig. 6, particularly along the plateau. However, the qualitative effect of increasing the PAB concentration is apparent.

Glycine was required in 15 times the concentration of PAB in order to

achieve maximum synthesis of PAH (Fig. 7). The presence of two linear slopes in the glycine concentration curve cannot be explained at this time.

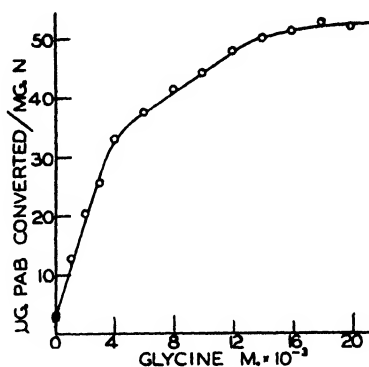


FIG. 7. Effect of varying glycine concentrations on the production of *p*-aminohippuric acid from *p*-aminobenzoic acid (PAB). 1.0 ml., containing 4.9 mg. of nitrogen, of a rat liver homogenate was added per flask. Final concentrations, 0.001 M PAB, 0.00078 M magnesium sulfate, 0.04 M potassium phosphate at pH 7.55. Total volume of 4.0 ml. made isotonic with KCl, incubated 45 minutes at 38° under 100 per cent oxygen.

TABLE II

Replacement of Glycine by Acetate or Glyoxylic Acid

1.0 ml., containing 5.1 mg. of nitrogen, of a rat liver homogenate was added per flask. Final concentrations, 0.001 M *p*-aminobenzoic acid (PAB), 0.00078 M magnesium sulfate, 0.045 M potassium phosphate at pH 7.55, 0.00012 M cytochrome *c*. Total volume of 4.0 ml. made isotonic with KCl. Incubated 45 minutes at 38° under 100 per cent oxygen. Total PAB-PAH recovered was 101 per cent.

Glycine concentration	Acetate concentration	Glyoxylate concentration	NH ₄ Cl concentration	PAB converted
$M \times 10^{-3}$	$M \times 10^{-3}$	$M \times 10^{-3}$	$M \times 10^{-3}$	γ per mg. N
5.0				27.7
	5.0			3.8
5.0	5.0			30.3
10.0				37.2
		5.0	5.0	3.3
5.0		3.8	3.8	12.5

Replacement of Glycine—If glycine is replaced by 0.005 M acetate (Table II), the synthesis of PAH drops to a value nearly as low as that corresponding to the production with no added glycine (Fig. 7). This failure of the N-acetylated derivative to support the synthesis is not due to any inhibitory effects, since, with added glycine in the presence of

aceturate, the level of production of PAH is even higher than with glycine alone, but it does not approach the value obtained with additional glycine.

Glyoxylate and ammonia likewise cannot be substituted for glycine in 0.005 M concentrations. However, in even lower concentrations, these compounds inhibit the formation from added glycine.

The magnitude of the total recoveries of the added PAB in this and other experiments precludes acetylation of the *p*-amino group in significant amounts in our studies, as was found in experiments with tissue slices (1).

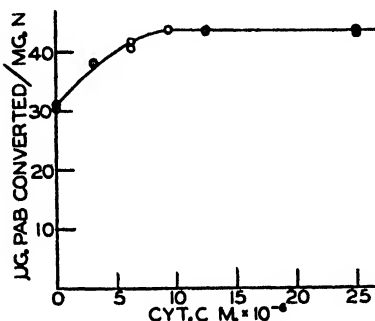


FIG. 8.

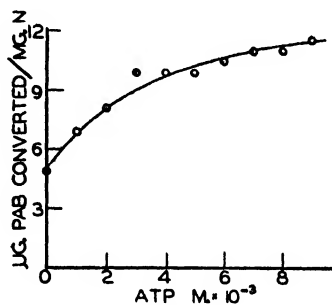


FIG. 9.

FIG. 8. The stimulation of the conversion of *p*-aminobenzoic acid (PAB) to *p*-aminohippuric acid by cytochrome *c*. 1.0 ml., containing 4.8 mg. of nitrogen, of a rat liver homogenate was added per flask. Final concentrations, 0.001 M PAB, 0.01 M glycine, 0.00078 M magnesium sulfate, 0.047 M potassium phosphate brought to pH 7.55. Total volume of 4.0 ml. made isotonic with KCl; incubated 45 minutes at 38° under 100 per cent oxygen.

FIG. 9. The support of anaerobic synthesis of *p*-aminohippuric acid from *p*-aminobenzoic acid (PAB) by adenosine triphosphate (ATP). 1.0 ml., containing 4.5 mg. of nitrogen, of a rat liver homogenate was added per flask. Final concentrations, 0.001 M PAB, 0.01 M glycine, 0.00078 M magnesium sulfate, 0.051 M potassium phosphate brought to pH 7.55. Total volume of 4.0 ml. made isotonic with KCl; incubated 45 minutes at 38° under 100 per cent nitrogen; Van Slyke oxygen-absorbing mixture (8) was placed in the center wells.

Cytochrome *c*—Cytochrome *c* is required to attain maximum synthesis, 0.000009 M being sufficient to saturate the system (Fig. 8). We have usually added 0.000012 M cytochrome *c* to our media to prevent deficiency of this substance being the limiting factor.

Adenosine Triphosphate—ATP in high concentrations will support the reaction anaerobically (Fig. 9), but the magnitude of the formation of PAH is low compared to that achieved aerobically. Since this low value might be due to the rapid hydrolysis of the ATP by adenosinetriphosphatase (ATPase), an experiment was devised in which ATP was added at varying time intervals, so that the final amount of ATP in each flask

was constant (Table III). Adding 12 micromoles of ATP initially, in two 6 micromole quantities, or in three 4 micromole quantities, achieved substantially the same small increase over the control level.

Since the possibility existed that fluoride exerted its inhibitory effect through a secondary action and fluoride is known to repress the hydrolysis of ATP, an experiment was run in which ATP was added anaerobically in the presence of fluoride (Table IV). ATP was without action aerobically, when it was added alone. Fluoride inhibits anaerobically even in 0.001 M concentrations, in which it was without effect aerobically (Fig. 4). Although ATP stimulated synthesis in the presence of fluoride, the level was below that attained in the absence of fluoride.

TABLE III

Effect of Successive Adenosine Triphosphate (ATP) Additions on Anaerobic p-Aminohippuric Acid Formation

1.0 ml., containing 5.5 mg. of nitrogen, of a rat liver homogenate was added per flask. Final concentrations, 0.001 M *p* aminobenzoic acid (PAB), 0.01 M glycine, 0.037 M potassium phosphate at pH 7.55, 0.00078 M magnesium sulfate. Total volume of 4.0 ml. made isotonic with KCl. Van Slyke oxygen-absorbing mixture (8) in the center wells. Incubated 15 minutes at 38° under 100 per cent nitrogen. Initial ATP added to the main compartment; successive portions tipped in from the side arms at stated times.

Time added <i>min</i>	ATP additions	PAB converted <i>γ per mg. A</i>
	Amount added <i>micromoles</i>	
0	12.0	6.5
0	6.0	8.0
5	6.0	8.1
0	4.0	
5	4.0	
10	4.0	8.9

The high ATPase activity that would be expected in the homogenate concentrations employed prevents an accurate assessment of the rôle of ATP from these experiments. However, the fact that ATP will support the reaction anaerobically, even though the magnitude of conversion is low, indicates that high energy phosphates are closely, if not directly, concerned in the formation of the peptide bond.

Cometabolites and Coenzymes—Various energy-yielding substrates and coenzymes were tested (Table V). Neither DPN nor thiamine pyrophosphate had any effect when added alone. Various components of the citric acid cycle, including all of the 4-carbon dicarboxylic acids, α -ketoglutarate, and citrate, stimulated the synthesis markedly. Glutamate and pyruvate

TABLE IV

Effect of Adenosine Triphosphate (ATP) in Presence of Fluoride on p-Aminohippuric Acid Formation

1.0 ml., containing 4.47 mg. of nitrogen, of a rat liver homogenate was added per flask. Final concentrations, 0.001 M *p*-aminobenzoic acid (PAB), 0.01 M glycine, 0.00078 M magnesium sulfate, 0.037 M potassium phosphate at pH 7.55 in all flasks. Fluoride added as sodium salt. Cytochrome *c*, 0.000012 M in flasks with oxygen. Total volume of 4.0 ml. made isotonic with KCl. Alkali was added to the center wells of aerobic flasks, and the Van Slyke oxygen-absorbing mixture (8), to the wells of anaerobic flasks. Incubated 45 minutes at 38°.

Gas phase	Fluoride concentration $M \times 10^{-4}$	ATP concentration $M \times 10^{-3}$	PAB converted γ per mg N
Oxygen			35.8
"		3.0	35.8
Nitrogen			7.5
"		3.0	9.5
"	1.0		5.0
"	5.0		3.6
"	1.0	3.0	8.3
"	5.0	3.0	7.8

TABLE V

Effect of Metabolites and Coenzymes on p-Aminohippuric Acid Formation

1.0 ml., containing 3.8 to 4.3 mg. of nitrogen respectively of a rat liver homogenate, was added to each flask of both experiments. Final concentrations, 0.001 M *p*-aminobenzoic acid (PAB), 0.015 M glycine, 0.00078 M magnesium sulfate, 0.037 M potassium phosphate at pH 7.55, 0.000012 M cytochrome *c*. Total volume of 4.0 ml. made isotonic with KCl. Incubated 45 minutes at 38° under 100 per cent oxygen.

Experiment No.	Metabolite	$M \times 10^{-4}$	PAB converted γ per mg V	per cent control
1	Glutamate	5.0	87.8	131
	α -Ketoglutarate	5.0	77.0	115
	Citrate	5.0	91.3	136
	Succinate	5.0	88.4	132
	Malate	5.0	93.9	140
	Oxalate	0.5	69.4	103
	Hexose diphosphate and 0.0005 M oxalate	5.0	66.2	99
	Coenzyme I	0.77	69.4	103
	Thiamine pyrophosphate	0.97	66.2	99
	Control		67.0	
	Succinate	5.0	79.8	144
2	Malate	5.0	87.5	160
	Oxalacetate	5.0	86.6	159
	Fumarate	5.0	89.0	162
	Pyruvate	5.0	74.2	131
	Control		54.6	

also caused an increased production. Oxalate, in the concentration present in the hexose diphosphate after removal of the calcium (see "Procedures and methods"), was without marked effect, and the hexose diphosphate was inactive. Although the absence of DPN might account for the failure of hexose diphosphate to show a stimulation, malate and glutamate were both active in the absence of added coenzyme. Of all of these additional substrates, succinate, malate, oxalacetate, fumarate, citrate, and glutamate were most effective in stimulating the reaction. Since the addition of glutamic acid introduces the complication of the possible formation

TABLE VI

Effect of Fumarate and Oxygen on p-Aminohippuric Acid Formation

1.0 ml., containing 5.8 mg of nitrogen, of a rat liver homogenate was added to each flask. Final concentrations, 0.001 M *p*-aminobenzoic acid (PAB), 0.015 M glycine, 0.00078 M magnesium sulfate, 0.000012 M cytochrome c, 0.037 M potassium phosphate at pH 7.55. Final volume of 4.0 ml. made isotonic with KCl. Incubated 45 minutes at 38° under 100 per cent oxygen.

Gas phase	Fumarate concentration	PAB converted
	$M \times 10^{-3}$	γ per mg. N
Oxygen		57.4
"	0.62	70.6
"	1.25	74.7
"	2.50	81.5
"	3.75	80.1
"	5.00	81.5
"	6.25	81.6
"	7.50	80.0
"	10.00	79.8
"	12.50	79.6
Air		66.9
"	12.50	80.0

of *p*-aminobenzoylglutamic acid, we eliminated it as a metabolite for further study, and of the remaining compounds chose fumarate as a representative 4-carbon acid for more intensive investigation.

Fumarate exerts its maximum action at the relatively low concentration of 0.0025 M (Table VI), and further increases in concentration are without action. The data also show that, in the absence of fumarate, lowering the oxygen tension by using air as the gas phase stimulates the reaction, while in the presence of fumarate this effect disappears.

The addition of 4 mg. of DPN or 4 micromoles of ATP in the presence of 0.0025 M fumarate stimulated the synthesis (Table VII) in contrast to the results obtained in the absence of fumarate. Surprisingly, the addition

of both DPN and ATP inhibited the formation of PAH (qualitatively it could be observed that the oxygen uptake was *greater* in the presence of both). While nicotinamide inhibited the synthesis, perhaps through a competitive formation of nicotinuric acid, the same effects with ATP

TABLE VII

Effect of Adenosine Triphosphate (ATP) and Coenzyme I (DPN) in Presence of Fumarate

1.0 ml., containing 4.3 mg. of nitrogen, of a rat liver homogenate was added per flask. Final concentrations, 0.001 M *p*-aminobenzoic acid (PAB), 0.015 M glycine, 0.0025 M fumarate, 0.000012 M cytochrome *c*, 0.00038 M magnesium sulfate, 0.056 M potassium phosphate at pH 7.55. Total volume of 4.0 ml. made isotonic with KCl. Incubated 40 minutes at 38° under air.

ATP concentration	DPN concentration	Nicotinamide concentration	PAB converted
$M \times 10^{-3}$	$M \times 10^{-3}$	$M \times 10^{-3}$	γ per mg. N
	1.54		71.6
		3.0	76.0
	1.54	3.0	55.5
2.0			64.1
2.0	1.54		81.5
2.0	1.54	3.0	61.3
			35.8

TABLE VIII

Effect of Varying Adenosine Triphosphate (ATP) Concentrations in Presence of Fumarate

1.0 ml., containing 4.04 mg. of nitrogen, of a rat liver homogenate was added per flask. Final concentrations, 0.001 M *p*-aminobenzoic acid (PAB), 0.015 M glycine, 0.0025 M fumarate, 0.000012 M cytochrome *c*, 0.00038 M magnesium sulfate, 0.056 M potassium phosphate at pH 7.55. Total volume of 4.0 ml. made isotonic with KCl. Incubated 40 minutes at 38° under air.

ATP concentration	PAB converted
$M \times 10^{-3}$	γ per mg. N
1.25	67.4
2.50	75.4
3.75	74.4
5.00	75.8
	76.1

and DPN could be observed in the presence of nicotinamide, which was added to inhibit the hydrolysis of the DPN (9). The amount of ATP required for maximum stimulation is less than 0.0013 M (Table VIII).

Homogenate Concentration—In the absence of fumarate, a pronounced

dilution effect was observed upon varying the amount of homogenate added (Fig. 10). An extrapolation of the linear portion of the curve intersected the zero conversion level at 2.5 mg. of homogenate nitrogen, and 12.7 mg. of nitrogen were required to effect complete conversion of the PAB to PAH in 45 minutes. With 0.0025 M fumarate present, as shown by the solid points of Fig. 10, the dilution effect largely disappeared, an extrapolation to zero intersecting the axis at 0.4 mg. of nitrogen, and 7.5 mg. of nitrogen were required to convert PAB to PAH completely in 45 minutes.

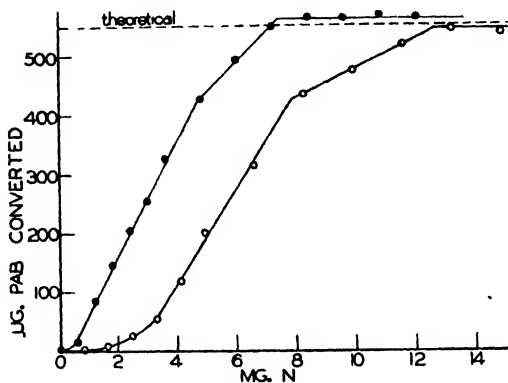


FIG. 10 Conversion of *p*-aminobenzoic acid (PAB) to *p*-aminohippuric acid by varying quantities of homogenate. Amount of homogenate expressed in terms of homogenate nitrogen. Final concentrations, 0.001 M PAB, 0.015 M glycine, 0.00078 M magnesium sulfate, 0.000012 M cytochrome *c* in all flasks. Experiments designated O contain no fumarate, 0.05 M potassium phosphate brought to pH 7.55, and were incubated under 100 per cent oxygen. Experiments designated ● contain 0.0025 M fumarate, 0.06 M potassium phosphate at pH 7.55, and were incubated under air. Total volume of 4.0 ml. made isotonic with KCl in all flasks; incubated 45 minutes at 38°.

Kinetics—With fumarate absent, the reaction proceeds linearly for 15 minutes, then changes slope and proceeds linearly at a lower rate until 40 minutes from zero time, and then stops completely (Fig. 11). (The time at which the reaction stops varied somewhat in livers from different animals.) In the presence of 0.0025 M fumarate, the reaction proceeds at a faster rate, continues linearly after the 15 minute change in slope until about 50 minutes have elapsed, again changes slope, and continues linearly. In an extension of the curve (not shown) in the presence of 0.005 M fumarate, these results were duplicated up to 60 minutes, indicating that the additional fumarate does not further stimulate up to 60 minutes. The linear portion after 50 minutes continued to 80 minutes, when a new slope appeared that continued to 100 minutes, the termination of the

experiment. At the 100 minute point, a conversion of 126 γ of PAB per mg. of homogenate nitrogen was attained.

A time study was also made in the presence of 0.0025 M ATP and 0.0025 M fumarate. While the rate was somewhat faster than with fumarate alone, the shape of the curve was substantially the same. It is remarkable that in all cases the rate of conversion changes after 15 minutes. This has been confirmed by running a rate study at 2.5 minute intervals over the first 30 minutes.

It is also noteworthy that, when fumarate is added, the activity of homogenates made from livers of different animals is strikingly consistent.

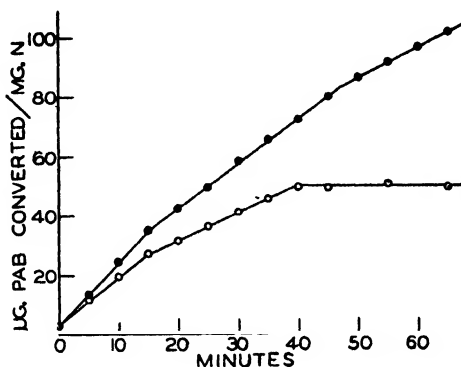


FIG. 11. Kinetics of the formation of *p*-aminohippuric acid from *p*-aminobenzoic acid (PAB). Total concentrations, 0.001 M PAB, 0.015 M glycine, 0.00078 M magnesium sulfate, 0.000012 M cytochrome *c*, 0.060 M potassium phosphate brought to pH 7.55 in all flasks. Total volume of 4.0 ml. made isotonic with KCl. Experiments designated ○ contained no fumarate, 1.0 ml. of rat liver homogenate, 4.7 mg. of nitrogen, and were incubated at 38° under 100 per cent oxygen. Experiments designated ● contained 0.0025 M fumarate, 1.0 ml. of rat liver homogenate, 4.8 mg. of nitrogen, and were incubated at 38° under air.

Values of 72.1, 67.5, 70.2, 71.3, 70.5, 71.8, and 67.4 γ of PAB converted per mg. of homogenate nitrogen were attained in 40 minutes in six consecutive experiments, with a different animal for each homogenate, an extreme variation of about 8 per cent and a mean deviation of ± 2.1 per cent.

The homogenate preparations are more active than were tissue slices (1). With homogenates, the Q_{PAH} is of the order of 1.7 for the 40 minute incubations, while, with slices, the value was 0.5 to 0.6.

DISCUSSION

The question as to whether the formation of PAH is a typical peptide bond synthesis or a specific instance still cannot, and need not, at present

be answered. Even though the synthesis may be catalyzed by enzymes specific for hippuric acid formation, the mechanism must be quite similar from an energetic standpoint, if not identical, to that required to acylate glycine with the α -amino carboxylic acids.

The results of this study demonstrate that the synthesis is closely associated with the normal energy-yielding metabolic pathways; *i.e.*, the citric acid cycle. Not only do the compounds associated with the cycle stimulate the production of PAH, but they are *required* in order to maintain the reaction at low tissue concentrations, where the endogenous metabolites are diluted to ineffective levels. Further, in the absence of any added metabolite, the synthesis abruptly stops after 40 minutes, but if fumarate is added, it can continue for at least 100 minutes. The maximum effective concentration of fumarate (0.0025 M) is low when added alone, and it is probable that the requisite concentration of the metabolites of the complete citric acid cycle, when all are present, is much lower, and may approach catalytic values. If this is true, the sudden cessation of the reaction at 40 minutes in the absence of fumarate can be understood, since it could represent the complete depletion of one or more essential metabolites in the catalytic cycle. Likewise, in the presence of fumarate, the changes in rate at the 50 and 80 minute times could represent the cessation of reactions not essential to the synthesis, but which contribute indirectly to the maintenance of the energy supply.

The change in rate at 15 minutes, however, is independent of the metabolites we have added, and no valid explanation for it is available. The elucidation of the actual mechanism of the synthesis will be necessary to clarify this question.

The failure of aceturic acid to replace glycine would indicate that the transacylation mechanism proposed by Rittenberg and Shemin (10) is not involved in this synthesis. The possible mechanisms involving oxidative deamination (11) can be excluded, not only because of the failure of glyoxylic acid and ammonia to participate in the synthesis, but also by the maintenance of anaerobic synthesis by ATP.

It is evident that the actual mechanism must invoke a means of transferring energy from the oxidative reactions of the citric acid cycle to the synthetic reaction by which PAH is formed. This transfer must in turn involve a compound common to the two systems. It is not likely that an enzyme is common to both reactions, and direct coupling is thus improbable. The remaining alternative is that the energy liberated during the oxidation of the citric acid cycle components is "stored" in some compound, which can react with glycine or PAB, creating a high energy intermediate whose energy is released upon the formation of the peptide bond. That the oxidation-reduction carriers, such as DPN, are the mediators is unlikely for the same reasons that oxidative deamination is unlikely. The most

obvious possibility is the formation of high energy-phosphorylated compounds, which react to yield either *p*-aminobenzoyl phosphate (acyl phosphate) or N-phosphoglycine (amide phosphate). The peptide bond would then be formed by the release of phosphoric acid. The oxidation of the citric acid cycle components is known to result in the formation of high energy phosphate, which can be stored as ATP or phosphocreatine (12-14). The phosphorylation of the amino acids might involve the transfer from the adenylic system or directly from such compounds as phosphopyruvic acid, or either.

The low magnitude of the maximum effective concentration of ATP in the presence of fumarate would indicate that it is playing a catalytic rôle in the reaction as a phosphate transfer system rather than having a direct function. This view is supported by the inability to reach the magnitude of aerobic synthesis anaerobically in the presence of ATP, although these observations are complicated by the ATPase activity to be expected in crude liver homogenates. If these observations are valid, then the activity of ATP could be accounted for by its function in transferring high energy phosphate to a more specific phosphate donor directly concerned in the peptide bond synthetic mechanism.

The stimulating action of DPN added alone is complicated by the presence of adenylic acid as an impurity in the preparation, which might account for the acceleration observed. However, this is not true of the inhibiting action of DPN in the presence of ATP, which is difficult to understand. The combination of the two substances apparently favors a competing reaction which interferes with the normal chain of energy-yielding oxidations by stimulating the oxidative metabolism and removing one or more components of the citric acid cycle, or some other key metabolite.

We propose to investigate the substitution of *p*-aminobenzoyl phosphate for *p*-aminobenzoic acid and N-phosphoglycine for glycine in a future study, which should establish whether either of these compounds is a direct intermediate in the synthesis of PAH.

Previous investigations by other workers have failed to demonstrate peptide bond synthesis in homogenates (15, 16). It is not clear why such attempts have failed, since complete details of the methods were not given. If the customary Krebs' Ringer's solution, which contains calcium ion and little potassium ions, was employed, such attempts would be negative. Gross differences in the hydrogen ion concentration would also prevent synthesis.

SUMMARY

1. The formation of *p*-aminohippuric acid (PAH) from *p*-aminobenzoic acid and glycine has been studied in rat liver homogenates.

2. The formation of PAH is stimulated by potassium and magnesium ions, and is inhibited by calcium and fluoride ions. Phosphate ion had no effect from 0.01 and 0.06 M.

3. The reaction is sensitive to pH changes and has a maximum rate at pH 7.55.

4. Adenosine triphosphate (ATP) supported the reaction anaerobically.

5. Cytochrome *c* stimulated synthesis of PAH aerobically.

6. Succinate, fumarate, malate, oxalacetate, citrate, α -ketoglutarate, glutamate, and pyruvate stimulated the reaction. Hexose diphosphate and thiamine pyrophosphate were without effect. ATP and coenzyme I (DPN) had no effect alone but stimulated the reaction in the presence of fumarate, although ATP and DPN together inhibited.

7. Fumarate had maximum action at 0.0025 M, and eliminated a marked dilution effect otherwise obtained. In the absence of fumarate, the formation of PAH ceased at 40 minutes, but, in its presence, the reaction continued beyond 100 minutes.

8. A mechanism is indicated in which synthesis occurs upon transfer of high energy phosphate, possibly to one of the reactants.

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ESTIMATION OF THE PHOSPHOLIPIDES IN HUMAN BLOOD

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Sphingomyelin is not affected by treatment with sodium alcoholate (1) or by potassium hydroxide (2) under conditions that will produce hydrolysis of glyceryl ester phosphatide. This resistance of sphingomyelin to mild alkaline hydrolysis has been used in its purification (1, 3, 4) and assay (2, 5). The present work demonstrates that the choline of lecithin is quantitatively liberated under the conditions of alkaline hydrolysis which permits assay of sphingomyelin. Extension of the latter method (2, 5) to include the present finding permits the estimation of lecithin and cephalin. The extended method has been applied to the analysis of blood phospholipides

EXPERIMENTAL

Method

4 ml. each of blood and plasma are placed in suitable containers, frozen with liquid air, and dried *in vacuo* while frozen (this material may then be kept indefinitely). 15 ml. of blood are sufficient for duplicate assays of blood and plasma and estimation of the cell volume. The concentration of cell phospholipides is estimated from the cell volume and the concentrations in blood and plasma.

The dried blood and plasma are separately extracted for 6 hours with 20 ml. of a mixture of equal parts of chloroform and methanol in a continuous extractor similar to one described by Thannhauser and Setz (6). To facilitate boiling and to inhibit oxidation the extraction flask should be equipped with a boiling tube through which nitrogen is bubbled. The extract is cooled, filtered, and made up to a volume of 25 ml. with additional solvent.

Total phosphorus (7) is determined on 1 ml. samples of this extract. A 10 ml. sample is placed in a suitable tube, heated in a water bath at 60°, and the solvent removed by a current of air. When cool, 5 ml. of N potassium hydroxide are added, the tube stoppered, and the mixture incubated at 37° for 16 hours. When the suspension has reached room temperature, a 1 ml. portion is acidified with 1 ml. of 1.5 N hydrochloric acid and 3 ml. of 0.5 M trichloroacetic acid. In order to obtain a permanently clear filtrate this acidified mixture must stand 60 minutes before filtration through Whatman No. 30 filter paper. 1 ml. of this filtrate is used for the determination of total phosphorus (7).

For the estimation of choline 1 ml. of 4.5 N hydrochloric acid is added to 3 ml. of the potassium hydroxide suspension. This mixture is let stand 60 minutes, after which it is filtered through asbestos with gentle suction and the filter washed twice with 1 ml. of 1.5 N hydrochloric acid. To the combined filtrate and washings are added 2 ml. of a freshly prepared saturated solution of ammonium reineckate in 0.5 N hydrochloric acid. After the solution has stood at 25° for 30 minutes, the glistening pink crystalline precipitate of choline reineckate is collected on a Pyrex M filter tube by means of gentle suction and washed twice with 2 ml. of ethanol. After the ethanol is removed by drawing air through the filter, the precipitate is dissolved in 3 ml. of acetone. The optical density of the solution is then determined in a Beckman quartz spectrophotometer at 526 m μ (slit width, 0.01 mm.) and the concentration of choline determined by interpolating from a standard curve.

The difference between total lipid phosphorus and that liberated by the alkaline hydrolysis is a measure of sphingomyelin. The choline liberated by the hydrolysis is a measure of lecithin. The phosphorus liberated by the hydrolysis not accounted for as lecithin is a measure of cephalins. Any acetal phosphatide present will appear in the cephalin fraction.

Extraction of Phospholipide—Mixtures of phospholipides such as those obtained from biological material and the individual phospholipides, except acetal phosphatide, are readily soluble in benzene. However, it was found that when plasma and serum, frozen dry, were extracted by hot benzene for 6 hours in the apparatus referred to above less than 0.5 per cent of the total lipid phosphorus could be demonstrated in the extract. In contrast about 10 per cent of the phospholipide of blood was so extracted; of this, three-fourths was cephalin, and the remainder was equally distributed between lecithin and sphingomyelin. On the other hand, extraction of the phospholipides from blood, plasma, and serum, frozen dry, by hot chloroform-methanol was found to be complete in 6 hours, since a second 6 hour extraction of the residue with fresh solvent yielded additional lipid phosphorus equivalent to less than 0.5 per cent of the total phospholipide. These observations suggest that the phospholipides of blood are primarily in the form of a phospholipide-protein complex which can be broken by polar solvents.

Determination of Choline—The conditions for quantitative determination of choline were studied. Maximum precipitation of choline reineckate was found to occur only when the ammonium reineckate solution was freshly prepared for each set of determinations; e.g., with ammonium reineckate solutions that had stood for 2 days at 25° about 30 per cent less choline was obtained from plasma lipides and from so-

lutions of choline than with freshly prepared ammonium reineckate solutions. Complete precipitation occurred within 30 minutes at 25°; no additional precipitate was formed on refrigeration at 0° for 60 minutes. Ethanol was used to remove excess Reinecke acid from the choline reineckate because it was found to be as effective as *n*-propanol and more volatile. Water was not used because choline reineckate is appreciably soluble in water; *e.g.*, a 10 mg. sample of choline reineckate, washed

TABLE I
Effect of Hydrolysis on Various Lipide Preparations

The data are expressed in mm per gm.

Lipide preparation	Total phosphorus	Hydrolyzed with N KOH at 37° for 16 hrs.		Choline liberated by 0.5 N Ba(OH) ₂ at 100° in 3 hrs.	Choline liberated by N KOH at 100° in 4 hrs.
		Acid-soluble fraction			
		Phosphorus	Choline		
A. Lecithin, CdCl ₂	0.94	0.92	0.860	0.860	0.865
B. " (beef heart)	1.19	1.05	0.875	0.865	0.860
C. Cephalin	1.20	1.19			
D. Lipositol	0.82	0.81			
E. Acetal phosphatide	2.40	2.32	0		
F. Sphingomyelin	1.25	0	0		
G. " reineckate	0.456	0.316	0.222		
(2 samples)	0.445	0.228	0.200		

Preparation A, from eggs according to Levene and Rolf (10); Preparation B, gift from Dr. Mary C. Pangborn; Preparation C, from human brain according to Folch (11); Preparation D, gift from Dr. D. W. Woolley; Preparation E, from beef brain according to Klenk (12) (the acetone-dried beef brains were generously supplied by The Wilson Laboratories, Chicago); Preparation F, from beef brain (1); Preparation G, each sample from a whole cat brain (6).

repeatedly with water, ethanol, and *n*-propanol, was then washed with 10 ml. portions of water at 25°. These aqueous washings contained choline reineckate equivalent to 23 γ of choline per ml.

Recovery of choline added to the filtered, acidified, hydrolysate prepared from extracts or of choline was satisfactory; *e.g.*, 86, 86, and 29 γ of choline were added to the prescribed aliquots of acidified hydrolysate which contained 174, 120, and 130 γ of choline respectively. The total amounts found were 254, 210, and 160 γ respectively. The treatment with alkali, under the conditions described, did not affect recovery of choline.

The standard curve was prepared by treating known amounts of choline (38 to 732 γ) as choline chloride (Pfanstiehl) in 5 ml. of water with 2 ml.

of a freshly prepared saturated solution of ammonium reineckate in 0.5 N hydrochloric acid. The precipitates of choline reineckate were collected, washed, and dissolved in acetone as described under "Method." The optical densities at 526 $m\mu$ (slit width, 0.01 mm.) were determined in the spectrophotometer. This standard curve was found to coincide with a curve obtained by measurement of acetone solutions of known concentration prepared from well washed choline reineckate. Because the

TABLE II

Phospholipide Composition of Blood from Normal Human Subjects

The blood samples, drawn from the median cubital vein and placed in a mixture of dry ammonium and potassium oxalates (13), were collected on different days, 3 hours after a breakfast of two eggs, milk, and cereal and 15 hours after the previous evening meal. The cell volume was determined by centrifugation (14). The mean molecular weight of the phospholipides is taken to be 800 and the concentrations are expressed in mm per liter.

Subject	Hrs of fasting	Material	Total phospholipide	Lecithin	Cephalin	Sphingo myelin	Ratio, choline phospholipide to total phospholipide	Cell volume
								<i>per cent</i>
Female, age 20 yrs.	3	Blood	3.26	1.53	1.06	0.67	0.67	44.5
		Plasma	2.75	2.22	0.10	0.43	0.96	
		Cells	3.90	0.67	2.24	0.99	0.42	
	15	Blood	3.45	2.06	0.64	0.75	0.81	46.0
		Plasma	2.69	2.22	0.11	0.36	0.95	
		Cells	4.35	1.88	1.25	1.22	0.71	
Female, age 28 yrs	3	Blood	3.50	1.89	0.84	0.77	0.76	45.0
		Plasma	3.18	2.44	0.18	0.56	0.94	
		Cells	3.88	1.21	1.64	1.03	0.57	
	15	Blood	3.25	1.54	0.96	0.75	0.70	47.0
		Plasma	3.02	2.25	0.25	0.52	0.91	
		Cells	3.50	0.75	1.75	1.00	0.50	

extinction coefficient has been reported (8) to be greater at 327 $m\mu$, measurements were also made at this wave-length. The data obtained demonstrated that although Beer's law held at 526 $m\mu$ it did not at 327 $m\mu$. Measurements have been made at the lower wave-length by others (8) over a narrower range of concentration than here reported, in which instance Beer's law does hold.

Effect of Hydrolysis on Various Lipide Preparations—Several phospholipide fractions were used to test the efficacy of the hydrolysis to yield acid-soluble phosphorus and choline. All of the fractions except the sphingomyelin and sphingomyelin reineckates were soluble in N potassium

hydroxide at 37°. The results appear in Table I and demonstrate complete liberation of phosphorus from lecithin, cephalin, and acetal phosphatide and of choline from lecithin.¹ This phosphorus was still in organic linkage, presumably glycerophosphate, since digestion with sulfuric acid was required in order to give maximum values. The sphingomyelin remained intact. Although the acetal phosphatides are also resistant to alkaline

TABLE III
Serum Phospholipides of Normal Human Subjects

The blood samples were collected under the conditions described in Table II, except that no anticoagulant was used. The concentrations are expressed in mm per liter.

Sex	Age	Hrs. of fasting	Total phospholipide	Lecithin	Cephalin	Sphingo myelin	Ratio, choline phospholipide to total phospholipide
	yr.s.						
Males	19	3	2.45			0.37	
		15	2.00	1.29	0.34	0.37	0.83
	20	3	2.49	1.91	0.12	0.46	0.95
		15	2.49	1.95	0.12	0.42	0.95
	24	3	2.60	2.22	0.00	0.38	1.00
		15	2.48	1.85	0.21	0.42	0.91
	25	3	2.94	2.14	0.44	0.36	0.85
		15	3.00	2.42	0.00	0.58	1.00
	29	3	2.73	1.88	0.45	0.40	0.83
		15	2.62	1.90	0.27	0.45	0.90
Females	24	3	3.30	2.74	0.00	0.45	1.00
		15	3.28	2.74	0.05	0.49	0.98
	24	3	3.25	2.48	0.18	0.59	0.94
		15	3.20	2.58	0.12	0.50	0.96
	27	3	2.60	2.50	0.00	0.10	1.00
		15	2.49	2.22	0.00	0.27	1.00
Average		3	2.79	2.26	0.17	0.39	0.95
		15	2.69	2.12	0.14	0.43	0.95

hydrolysis (9, 12), they do not interfere with the determination of sphingomyelin since they are extremely labile in acid (9); as may be seen by the data in Table I the phosphorus is quantitatively recovered in the acid-soluble fraction after hydrolysis with N potassium hydroxide. The

¹ The fact that the phosphorus to choline ratio of Preparations A and B is not 1 indicates that the samples were not pure lecithin. The beef heart lecithin (Preparation B) contained 0.142 mm of amino nitrogen per gm., presumably from contamination with non-choline phospholipide.

results obtained with the sphingomyelin reineckates offer additional evidence to that previously reported (3) that sphingomyelin reineckate from crude lipid extracts is a lipid mixture, in this case containing sphingomyelin, lecithin, and cephalin.

Results

The results of the analyses on human blood, plasma, and serum are summarized in Tables II and III. Comparison with work of other investigators may be made from Table IV. As may be seen, the finding of Taurog *et al.* (18) that the phospholipides of plasma are predominantly choline-containing (*i.e.* lecithin and sphingomyelin) is confirmed by our studies on plasma and serum. The distribution of the phospholipides

TABLE IV
Blood Phospholipides Reported by Others

The original data have been converted to mm per liter.

Investigator	Material	No. of subjects	Total phospholipide	Lecithin	Cephalin	Sphingomyelin	Ratio, choline phospholipide to total phospholipide
Ramsay and Stewart (15)	Blood	12	3.52	0.41	0.82	2.29	0.76
Thannhauser <i>et al.</i> (16)	Serum	6	2.82	1.34	1.20	0.29	0.58
Kirk (17)	Plasma	20	1.81	0.24	0.85	0.72	0.53
	Cells	20	2.45	0.40	1.46	0.59	0.40
Taurog <i>et al.</i> (18)	Plasma	11	2.94		0.08		0.98

of serum was found to be the same as in plasma. Cephalin is shown to be the largest phospholipide fraction in cells and the sphingomyelin concentration of cells to be twice that of plasma. With respect to the present data no obvious correlation of the phospholipide composition of blood, plasma, serum, or cells with the length of fast could be established. It may be pointed out that the results summarized in Table IV were obtained by dissimilar methods. Duplicate assays were made throughout and agreed within ± 1 per cent for both phosphorus and choline.

SUMMARY

A method is described permitting determination of the phospholipide composition of plasma and cells on 15 ml. of blood in duplicate, agreeing within ± 1 per cent.

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THE RÔLE OF PYRIDINE NUCLEOTIDES IN THE REDUCTION OF METHEMOGLOBIN

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In the course of investigations concerning the use of *p*-aminopropiophenone-induced methemoglobinemia in the prevention of cyanide poisoning (1) and of the physiological impairments incident to methemoglobinemia (2, 3), it became of interest to study the mechanism of action between methylene blue and hemoglobin or methemoglobin. It has been shown, on the one hand, that methemoglobin is produced when methylene blue is injected into the normal organism (4, 5). On the basis of this action, methylene blue has been classified as a methemoglobin former and recommended as an antidote in the treatment of cyanide poisoning (5). On the other hand, it has also been demonstrated that the reduction of methemoglobin is accelerated when the dye is injected into the methemoglobinemic animal (6, 7). The present study is concerned with a further analysis *in vitro* of the reduction of methemoglobin in intact methemoglobinemic erythrocytes and in hemolysates, and with the possible rôle of pyridine nucleotides in this reduction.

EXPERIMENTAL

Methemoglobinemic cells were prepared *in vivo* by the injection of *p*-aminopropiophenone (2.5 to 3.0 mg. per kilo in 2 to 3 cc. of redistilled propylene glycol) into dogs. After approximately 1 hour most of these dogs exhibited symptoms of methemoglobin poisoning, and their erythrocytes showed a conversion of 60 to 80 per cent of their pigment to methemoglobin. Approximately 20 to 30 cc. of blood from normal or methemoglobinemic dogs were withdrawn by heart puncture and heparinized. The centrifuged cells were washed twice with 4 or 5 volumes of Ringer-phosphate solution of the following composition: 0.13 M NaCl, 0.0095 M CaCl₂, 0.0031 M KCl, 0.0015 M MgSO₄, and 0.01 M phosphate buffer, pH 7.40. After two such washings, glucose could no longer be detected. The cells were then

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diluted to the original blood volume with Ringer-phosphate solution and filtered through gauze to prevent the accidental introduction of small clots.

Hemolysates were prepared by centrifuging the normal or methemoglobinemic washed cell suspensions and removing the supernatant solution. The cells were frozen in an ice-salt mixture with continuous stirring, and appropriate amounts of precooled water were added. In certain experiments, hemolysis was performed in the presence of nicotinamide so as to yield a final concentration of 0.16 M nicotinamide in the hemolysate. The cells were immersed in a water bath at about 30° and thawed with constant stirring. After standing at room temperature for 30 minutes, the solutions were again centrifuged for 15 minutes and the clear supernatant liquid was withdrawn for use in the experiments.

Purified methemoglobin was prepared from guinea pig blood by the method of Warburg and Reid (8) and dialyzed at 0–4° against distilled water until nitrites could no longer be detected in the methemoglobin solution. The solution was diluted to a suitable volume with 0.05 M phosphate buffer (pH 7.40) and kept at 0–4° until use.

Sodium lactate solution was prepared by neutralizing lactic acid, c.p., with NaOH to phenol red. This solution showed no optical activity and was considered to consist of racemized sodium lactate. Sodium hexose diphosphate was obtained from the calcium salt (Schwarz) by treatment with the calculated amount of sodium oxalate and addition of a few drops of dilute CaCl_2 solution to the filtrate to insure the absence of oxalate ions. Methylene blue (U. S. P.), nicotinamide (U. S. P.), and dextrose (anhydrous, c.p.) were used.

Diphosphopyridine nucleotide (DPN) was prepared from pressed bakers' yeast according to the directions of Williamson and Green (9). The purity of the preparation was determined spectrophotometrically by measuring the change in absorption upon treatment with $\text{Na}_2\text{S}_2\text{O}_4$ at 340 $\text{m}\mu$, and estimated to be about 10 to 20 per cent. Since no standard samples of DPN were available, and only a first approximation of the purity was required, this method was chosen because of its convenience. Reduced diphosphopyridine nucleotide (DPN- H_2) was prepared according to Green and Dewan (10) except that $\text{Na}_2\text{S}_2\text{O}_4$ in the solid state rather than in solution was added to the DPN solution. The excess $\text{Na}_2\text{S}_2\text{O}_4$ was destroyed by passing oxygen in a vigorous stream through the solution for 30 minutes. If the solution was cloudy after the treatment with $\text{Na}_2\text{S}_2\text{O}_4$, it was centrifuged and the supernatant filtered.

The erythrocyte suspensions, hemolysates, or methemoglobin solutions, prepared as described above, were mixed in equal proportions with solutions of the various substrates in 25 or 50 cc. Erlenmeyer flasks. The flasks were immersed in a water bath at 38° and shaken at the rate of 110 oscillations

per minute. Duplicate samples for the analytical determinations were withdrawn from the reaction mixtures at the times stated in Tables I to III. Methemoglobin was determined spectrophotometrically in duplicate aliquots, immediately upon withdrawal of the sample, by a slight modification of the method of Evelyn and Malloy (11). The presence of methylene blue in the concentrations used in the experiments was found not to interfere with the determination of methemoglobin. Lactic acid was determined by the method of Barker and Summerson (12), and the amount of total blood pigment iron according to the method of Drabkin (13).

Results

Oxidation of Hemoglobin and Reduction of Methemoglobin by Methylene Blue—Warburg, Kubowitz, and Christian (14), using the evolution of oxygen as a measure of methemoglobin formation, found that methylene blue oxidized hemoglobin to methemoglobin in intact, washed erythrocytes or in hemolysates prepared from them, but that in the presence of added glucose methylene blue reduced methemoglobin in intact erythrocytes. These findings were confirmed and extended in the present study with the spectrophotometric technique. It was found that, if the reaction was permitted to proceed for a sufficient length of time, complete oxidation of hemoglobin by methylene blue occurred both in hemolysates and in washed intact erythrocytes. Thus, at a concentration of 0.27 mM methylene blue, 72 per cent of the blood pigment from washed and laked erythrocytes was oxidized to methemoglobin in 4 hours, 94 per cent in 24 hours, and 100 per cent in 44 hours at 38°. In the presence of glucose, oxidation by methylene blue was retarded in intact erythrocytes but not in hemolysates.

The addition of glucose or lactate to methemoglobinemic cells caused reduction, provided that methylene blue was present and the cells were intact. It may be mentioned that glucose was more effective than twice its molar concentration of *dl*-lactate. Thus, in a typical experiment, the concentration of methemoglobin decreased in 2 hours from an initial value of 84 to 28 per cent in the presence of 56 mM glucose and 0.13 mM methylene blue; in the presence of 110 mM *dl*-lactate and the same concentration of methylene blue, the methemoglobin concentration decreased to only 48 per cent. No reduction was observed in hemolysates from methemoglobinemic cells, prepared as described above, in the presence of methylene blue and either glucose, lactate, or hexose diphosphate.

The results of Warburg *et al.* (14) as well as those obtained in the present study suggest that during hemolysis a factor, essential in the reduction of methemoglobin in the presence of substrate and methylene blue, is lost. That the process of glycolysis is linked with the reduction of methemoglobin is implied by the work of Kiese (15) and of Drabkin (16) who showed that

iodoacetate and fluoride inhibit this reduction. In confirmation of their results, it was found that a concentration of 100 mM fluoride or 1 mM iodoacetate completely suppressed the reduction in the presence of glucose and methylene blue.

Although glycolysis appeared to be necessary for the reduction of methemoglobin, the extent of the reduction did not parallel the extent of lactic acid formation when glucose was used as substrate. Thus, in a typical experiment no methemoglobin was reduced and 9.3 micromoles of lactic acid were formed in 10 cc. of reaction mixture during 100 minutes in the absence of methylene blue; in the presence of 0.0013 mM methylene blue, 4.2 micromoles of methemoglobin were reduced but the amount of lactic acid formed was essentially the same, 8.7 micromoles. These results indicate that the connection between glycolysis and methemoglobin reduction in the presence of methylene blue does not consist of a simple interaction between glucose or lactate and methylene blue.

Rôle of Pyridine Nucleotides in Reduction of Methemoglobin—In view of the above results, it was considered possible that the lack of reduction of methemoglobin by methylene blue in hemolyzed cells was caused by the absence of pyridine nucleotides from hemolysates, since there is evidence that pyridine nucleotides disappear rapidly upon lysis of erythrocytes (17) and homogenization of several animal tissues. The disappearance may be counteracted by the addition of nicotinamide during the process of disintegration of the cell structure (18, 19). Consequently, the effect of nicotinamide, present at the time of hemolysis, upon the reduction of methemoglobin was tested.

Table I shows the extent of reduction of methemoglobin in hemolysates in the presence of nicotinamide and various substrates. No significant reduction occurred in the presence of nicotinamide and methylene blue when glucose was used as a substrate. With hexose diphosphate, 5 to 13 (average 9) per cent of the methemoglobin originally present was reduced in 1 hour when nicotinamide but no methylene blue was present. The addition of methylene blue to this system caused a much more rapid reduction; in nine experiments, an average of 33 per cent of the methemoglobin originally present was reduced in 1 hour under these conditions. This reduction was completely inhibited by the addition of 1 mM iodoacetate. The presence of nicotinamide also led to a substantial reduction of methemoglobin when lactate was chosen as the substrate.

The above data indicated that either DPN or triphosphopyridine nucleotide or both were necessary for the reduction of methemoglobin by lactate or hexose diphosphate. The reaction mechanism was assumed to consist of a reduction of pyridine nucleotides by these substrates, and a subsequent interaction between the reduced pyridine nucleotide and met-

TABLE I

Effect of Nicotinamide (NCA) and Methylene Blue (MB) on Reduction of Methemoglobin in Hemolysates with Glucose, Lactate and Hexose Diphosphate (HDP) As Substrates

The initial concentration of methemoglobin was 60 to 80 per cent of the total blood pigment

Fraction of methemoglobin reduced						
During 60 min. in presence of					During 120 min. in presence of	
2.8 mm glucose, 0.13 mm MB	160 mm NCA, 2.8 mm glucose, 0.13 mm MB	2.8 mm HDP, 0.13 mm MB	160 mm NCA, 2.8 mm HDP	160 mm NCA, 2.8 mm HDP, 0.13 mm MB	20 mm lactate, 0.13 mm MB	160 mm NCA, 20 mm lactate, 0.13 mm MB
per cent	per cent	per cent	per cent	per cent	per cent	per cent
0	0	0	5	27	2	7
3	2	0	7	31	0	18
3	4	0	9	31		20
		1	11	32		
			13	33		
				33		
				36		
				36		
				39		

TABLE II

Reduction of Purified Methemoglobin (MetHb) by Reduced Diphosphopyridine Nucleotide (DPN H₂) in Presence of Methylene Blue (MB)

The composition of the reaction mixture is expressed in terms of the absolute amounts of reactants, in micromoles, present in a volume of 6 cc. The concentration of methemoglobin is expressed as per cent of total blood pigment

Composition of reaction mixture		Concentration of methemoglobin after incubation at 38° C.		
		0 min	15 min	20 min
		per cent	per cent	per cent
0.044 MetHb*		90	90	
0.044	+ 0.29 DPN H	90	83	
0.044	+ 0.29 + 0.027 MB	90	12	
0.044	+ 0.29 DPN + 0.027	90	90	
0.068		98		98
0.068	+ 0.44 DPN H	98		85
0.068	+ 0.44 + 0.027 MB	98		8

* The controls are treated with a solution which contained the same amount of Na₂S₂O₄ as was used in the reduction of DPN and which had been exposed to oxygen for 30 minutes. The amounts of DPN and DPN H₂ given in the table are estimated on the basis of spectrophotometric assay of the preparation as described in the text.

hemoglobin, mediated by methylene blue. Table II shows the reaction of DPN-H₂ with purified methemoglobin, in the presence of methylene blue. It may be seen that methemoglobin was reduced to some extent directly by DPN-H₂ and that this reaction was greatly accelerated in the presence of methylene blue. No reduction occurred when DPN instead of DPN-H₂ was used. The rate of reduction of methemoglobin in this system was rapid; no further change in the concentration of methemoglobin was found after incubation for approximately 15 minutes at 38°. Table III shows that the extent of reduction of methemoglobin under these conditions was a function of the amount of reduced pyridine nucleotide added to the system.

TABLE III

Effect of Concentration of DPN-H₂ on Extent of Methemoglobin Reduction

Each reaction mixture contained 0.045 mm methylene blue. A relative concentration of DPN-H₂ of 1 represented a concentration of 0.017 mg. of a reduced Williamson-Green preparation in 6.0 cc. of reaction mixture.

Relative concentration of DPN-H ₂	Fraction of MetHb reduced in 30 min.
<i>units</i>	<i>per cent</i>
1	28
2	40
4	45
11	68
16	75
20	79

DISCUSSION

The present work has shown (a) that when nicotinamide, a known suppressor of pyridine nucleotide hydrolysis, is present hexose diphosphate or lactate can serve as substrate in the reduction of methemoglobin in hemolysates as well as in intact erythrocytes, while glucose is no longer utilized after hemolysis, and (b) that methemoglobin interacts with DPN-H₂ to yield hemoglobin. Both of these reactions are greatly accelerated by methylene blue. The findings indicate that, incident to hemolysis, processes other than destruction of pyridine nucleotides occur which abolish glycolysis in hemolysates. In view of the non-utilization of glucose in the reduction of methemoglobin in such a system it appears likely that one or more steps in the phosphorylating mechanism, leading to the production of hexose diphosphate from glucose, are abolished by hemolysis.

It is implied by the experiments here presented that the component in the Williamson-Green yeast preparation, active in the reduction of methemoglobin, is DPN-H₂. It is recognized that this preparation contains

substances other than DPN. However, several lines of evidence, derived from this study and the work of other investigators, support the conclusion previously expressed. Runnstroem, Lennerstrand, and Borei (20) observed that the addition of DPN to a system consisting of hemolyzed blood, hexose monophosphate or diphosphate, and methylene blue increased oxygen consumption and phosphorylations. Lennerstrand (17) reported that pyridine nucleotides of erythrocytes disappeared rapidly following lysis. Mann and Quastel (18) and Handler and Klein (19) demonstrated that nicotinamide in high concentrations was a specific competitive inhibitor of a nucleosidase which liberates nicotinamide from DPN during the disintegration of animal tissues. In the present study, reduction of methemoglobin in hemolysates proceeded only on addition of nicotinamide to the system during lysis. This reduction was abolished by the addition of low concentrations of iodoacetate, a known inhibitor of dehydrogenases which bring about reduction of DPN. The above findings indicate very strongly that the component in the Williamson-Green preparation, which, after treatment with $\text{Na}_2\text{S}_2\text{O}_4$, is active in methemoglobin reduction, is indeed DPN.

It appears that the essential feature of the reduction of methemoglobin within the intact erythrocyte or in solution is the interaction between DPN- H_2 and methemoglobin. Methylene blue mediates this reaction; it may be presumed to be reduced by DPN- H_2 to leucomethylene blue, which in turn reduces methemoglobin to hemoglobin. For the reducing action of methylene blue a continuous source of DPN- H_2 must thus be available. The removal of substrate, or the inhibition of glycolysis by fluoride or iodoacetate, interferes with the production of DPN- H_2 ; hemolysis results in the enzymatic destruction of DPN. If the formation of DPN- H_2 is prevented by any one of these means, leucomethylene blue is no longer regenerated in this system, and methylene blue acts purely as an oxidant.

SUMMARY

Reduction of methemoglobin by hexose diphosphate or lactate and methylene blue in hemolysates occurs only in the presence of nicotinamide during lysis. Glucose cannot function as substrate in this system, but is utilized in intact methemoglobinemic cells.

Addition of a partially purified preparation of reduced diphosphopyridine nucleotide from yeast to methemoglobin and methylene blue results in the rapid reduction of methemoglobin. Diphosphopyridine nucleotide in its oxidized form is ineffective in this system.

Conditions are defined for the action of methylene blue as an oxidant of hemoglobin or a reductant of methemoglobin.

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THE COLORIMETRIC DETERMINATION OF STREPTOMYCIN IN CLINICAL PREPARATIONS, URINE, AND BROTH*

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The streptomycin molecule contains a number of functional groups which may serve as bases for chemical assay. The Sakaguchi reaction has been used to test for the guanido groups of the molecule (1), and a reaction given by the N-methyl-L-glucosamine moiety (2) of streptomycin has been reported (3). Furthermore, the molecule has a free or potentially free carbonyl group (4) and marked reducing properties. Any of these groups can be used as a basis for assay purposes on highly purified material, but these reactions are not sufficiently specific for samples of lower potency or biological fluids.

A means of assay was suggested by the observation that streptomycin on being heated with dilute alkali forms maltol, 2-methyl-3-hydroxy- γ -pyrone (5). Maltol shows characteristic absorption maxima in the ultra-violet in both acid and alkaline solutions, and reacts with ferric ions to give a purple-red color which is stable in acid solution (6). We have further observed that maltol reacts with the phenol reagent of Folin and Ciocalteu to give the usual blue color. The colorimetric assay procedure for streptomycin presented in this paper is based on these reactions of maltol.

Determination of Streptomycin in Clinical Preparations

Reagents—

1 N and 2 N sodium hydroxide.

20 per cent sodium carbonate.

1 per cent ferric ammonium sulfate in 0.75 N sulfuric acid.

Phenol reagent (according to Folin and Ciocalteu (7)).

Maltol Formation—To 5 ml. of the streptomycin solution in a test-tube add 1 ml. of 2 N NaOH. Immerse the tube in a boiling water bath for 3 minutes and cool for 3 minutes in cold water.

Color Reactions—Either ferric ammonium sulfate or the phenol reagent is used to develop a color with the maltol, depending on the amount of streptomycin originally present. The sensitivity with the ferric ammonium sulfate reagent is 500 to 2500 γ of streptomycin; with the phenol reagent, 20 to 250 γ .

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Ferric Ammonium Sulfate Reagent—To the cooled alkaline solution add 4 ml. of the ferric ammonium sulfate reagent. Shake vigorously and determine the intensity of the purple color in a photoelectric colorimeter with Filter 540 after 10 minutes. The light transmittance of the blank is nearly the same as for water and is constant, varying not more than ± 0.5 deflection in the Evelyn photoelectric colorimeter.

Phenol Reagent—To the cooled alkaline solution add dropwise 1 ml. of the phenol reagent and mix well. Let stand 1 to 2 minutes, add 3 ml. of 20 per cent sodium carbonate, and shake vigorously. Read after 10 minutes in a photoelectric colorimeter with Filter 660. A blank is prepared simultaneously. Clinical preparations of streptomycin contain small quantities of material which reacts directly with the phenol reagent. Ordinarily this accounts for 2 to 5 per cent of the color value obtained after maltol formation. Therefore an additional determination is carried out with 5 to 10 times the quantity of the sample in exactly the manner described above except that the heating period with alkali is omitted. Since the formation of maltol from streptomycin occurs at an appreciable rate even at room temperature, the phenol reagent must be added *immediately* after the addition of the alkali.

Methanolic Solutions of Streptomycin—If streptomycin is to be determined in methanolic solution, the ferric ammonium sulfate reagent is applicable. Starting with 4 ml. of methanol solution in a 10 ml. graduated cylinder, add 2 ml. of 1 N NaOH and immerse the cylinders in a water bath at $65^{\circ} \pm 1^{\circ}$ for 20 minutes. After cooling add ferric ammonium sulfate reagent to the 10 ml. mark. The intensity of the purple color is measured after 10 minutes.

Calibration In this paper the accepted definition is used; namely, that 1 unit of streptomycin is equal to 1 γ of streptomycin-free base.

A sample of crystalline streptomycin trihydrochloride calcium chloride double salt (8) was the standard used in the calibrations. Since the salts of streptomycin are hygroscopic and vigorous drying may cause some decomposition, two samples are weighed out at the same time; one is used for a moisture determination by drying for 3 hours at 100° over phosphorus pentoxide *in vacuo*; the other is used for the calibration and a correction is made for the water content.

DISCUSSION

The *specificity* of the maltol formation on short heating with alkali has been investigated with a number of derivatives and cleavage products of streptomycin. Dihydrostreptomycin, obtained by reduction of the carbonyl group of the molecule, is biologically active, but does not form maltol (9). Neither N-methyl-L-glucosamine nor streptidine yields maltol. The

former is inactive by microbiological test and the latter shows only slight activity when compared to streptomycin. Aqueous acid hydrolysis of the glycosidic linkage between streptidine and the rest of the molecule results in the formation of a cleavage product, streptobiosamine (10), which does not form maltol. However, hydrolysis of streptomycin in methanolic hydrochloric acid and subsequent acetylation yield methyl tetraacetyl-streptobiosaminide dimethyl acetal (11) in which the streptidine is replaced by a methyl group in the glycosidic linkage. This product forms maltol on heating with alkali. Therefore, the maltol is derived from the center portion of the molecule, streptose (12), which must contain an intact car-

TABLE I

Specificity of Reagents Used for Determination of Maltol Formed from Streptomycin

The compounds listed were treated with alkali under the conditions of the test, and color development with the reagents was then obtained.

Substance, 10 mg	Phenol reagent	Ferric reagent	Substance, 10 mg	Phenol reagent	Ferric reagent
Glucose	++++	—	Hydroxyproline	—	—
Galactose	++++	—	Tyrosine	++++	—
L-Xylose	++++	—	Tryptophane	++++	—
L-Arabinose	++++	—	Phenol	++++	—
Sucrose	—	—	α -Naphthol	++++	—
Brown sugar	+	—	Resorcinol	++++	—
Maltose	++++	—	Pyrocatechol	++++	—
Lactose	++++	—	Pyrogallol	++++	—
Soluble starch	++++	—	Salicylic acid	++++	++++
Glycogen	—	—	Dihydrostreptomycin	±	—
D-Glucosamine	++++	—	Penicillin G (benzyl-penicillin)	—	—
N-Methyl-L-glucos	++++	—			
Streptidine					

bonyl group and apparently must be glycosidically linked to another group (10).

None of the compounds listed in Table I form maltol under the conditions of the test, as is shown by the absence of the characteristic absorption in the ultraviolet region. However, the specificity of the estimation of maltol by color development is limited by the reagents. The phenol reagent is, of course, quite unspecific, reacting with alkali degradation products of carbohydrates as well as with phenols. It should be pointed out that the N-methyl-L-glucosamine after heating with alkali gives some reaction with this reagent and the color formed with streptomycin will therefore be a summation of the one due to maltol formation and this side reaction. The phenol reagent, because of its unspecific nature, can only

be used on clinical samples or after previous separation of the maltol from the bulk of the interfering substances.

The ferric ammonium sulfate reagent is quite specific. It does not react with the carbohydrate degradation products and ordinary phenols fail to give any color reaction, since the final solution is acid. Acidic phenols like salicylic acid interfere. The disadvantage of the maltol-ferric ion reaction is its relatively low sensitivity.

The rate of maltol formation from streptomycin at the alkali concentration employed depends on the temperature. The maximum amount of maltol is formed at room temperature in 6 to 8 hours, at 65° in 15 minutes, and at 100° in 1 minute. No destruction of maltol occurs with the concentration of alkali used at 100° within 7 minutes and at 65° within 45 minutes.

TABLE II
Extent of Maltol Formation

From the molecular extinction coefficients of pure maltol and maltol formed from streptomycin.

	Ultraviolet absorption at 275 $m\mu$ E_m	Ferric ammonium sulfate reagent color at 540 $m\mu$ E_m	Phenol reagent color at 660 $m\mu$ E_m
Maltol	8880	1040	8300
“ from streptomycin	6730	768	8340
“ formed per mole streptomycin, moles	0.76	0.71	0.74*

* The actual color developed corresponds to 1.01 moles. Correction has been made for the color contributed by the alkali degradation product of N-methyl-L-glucosamine. (See the text.)

The extent of maltol formation was determined by comparing the molecular extinction coefficient of crystalline maltol and of streptomycin after heating with alkali, by means of the ultraviolet absorption at 275 $m\mu$ in 0.1 N hydrochloric acid and the two color reactions (Table II). In every case 0.75 mole of maltol is formed per mole of streptomycin. The apparently higher yield with the phenol reagent is due to the fact that the N-methyl-L-glucosamine part of the molecule forms some alkaline degradation product which also reacts. If correction is made for this unspecific color development, the maltol yield is calculated to be 0.74 mole.

The *reproducibility* of the method with either reagent is excellent. 95 determinations on the standard sample by four different operators at widely scattered times agreed within ± 3 per cent; thirty-four were in error less than ± 1 per cent, twenty-nine less than ± 2 per cent, and thirty-two less than ± 3 per cent.

Clinical samples of any potency were assayed at three different levels

by each of the two reagents. All six results agreed within the stated limit of error of ± 3 per cent.

Comparison with Microbiological Assay—The chemical assay values are in good agreement with the microbiological assay on clinical samples having potencies greater than 450 γ per mg. The per cent deviation of the microbiological assay from the chemical assay was determined in a series of 80 samples of clinical preparations of several salts of streptomycin. 67 per cent of the chemical assays agree within ± 5 per cent and 95 per cent within ± 10 per cent with the corresponding microbiological assays.

Good agreement with the microbiological assay is usually obtained with samples of potency below 450 γ per mg. However, in 20 to 30 per cent of these samples the chemical values were 10 to 50 per cent higher than in the corresponding microbiological assay. The bioassay values were obtained by averaging the results of a relatively small number of determinations which varied considerably, and some of the individual bioassays were in agreement with the chemical value. If this discrepancy is real rather than apparent, it could be interpreted to mean that not all of the streptomycin present is active in the biological test, or that in the chemical test interfering substances are reacting as streptomycin. In order for a substance to interfere, it must fulfil the high requirements of specificity previously discussed.

Determination of Streptomycin in Broth and Urine

The determination of streptomycin in broth and urine is also based on maltol formation. The relatively small quantities of streptomycin in these fluids require the more sensitive phenol reagent. The unspecific nature of the reagent and the presence of pigments necessitate the separation of the maltol from the bulk of the interfering materials prior to the color reaction. Such a separation is accomplished by extracting the maltol from acid solution with chloroform (two extractions with an equal volume suffice), and then reextracting from the organic layer with dilute alkali.

Normal urine and unfermented broth contain small amounts of interfering materials which are extracted in such a procedure and which react with the phenol reagent. Appropriate blanks, in which the streptomycin is removed without changing the quantity of interfering material, correct for these substances. Boiling in 1 N hydrochloric acid destroys streptomycin rapidly: both microbiological activity and ability to form maltol are lost within 5 minutes. In the case of broth an acid-pretreated aliquot can be used as an appropriate blank.

With urine, however, heating with acid hydrolyzes substances such as conjugated phenols and liberates considerable quantities of interfering material, making this procedure useless. Adsorption by Lloyd's reagent

accomplished the removal of streptomycin from urine without appreciably changing the quantity of interfering materials, and pretreatment of an aliquot of the urine with this adsorbent was used to obtain the necessary blank.

The amount of the interfering substances can be decreased by about one-half if the chloroform layer containing the maltol is washed once with an equal volume of a phosphate buffer of pH 7 saturated with sodium chloride. No maltol is removed from the chloroform by this step. Such a procedure is always necessary in urine, and in broth whenever more than 1 ml. is used for the assay.

Certain precautions are necessary in the extraction procedure to prevent too low assay values due to the deleterious effect of traces of oxidizing agents. The chloroform used for extraction is washed each day, all glassware is rinsed thoroughly from traces of cleaning fluids, the distilled water must be free from chlorine, and the length of time for the assay is limited.

Determination in Urine

Reagents—

2 N and 3 N sodium hydroxide.

20 per cent sodium carbonate.

Phenol reagent (7), diluted 1:1 with water.

Lloyd's reagent (Eli Lilly and Company).

1.5 N sulfuric acid.

Phosphate buffer, pH 7, saturated with sodium chloride. To 2 liters of 0.2 M secondary sodium phosphate add 28 ml. of 0.1 M citric acid and 690 gm. of sodium chloride.

Chloroform, reagent grade. Wash daily in the following manner: once with an equal volume of approximately 0.5 N sodium hydroxide and three times with an equal volume of distilled water. Dry with anhydrous sodium sulfate.

Procedure—Adjust the urine sample to pH 5.0 to 5.2 with a drop or two of glacial acetic acid, shake with an equal volume of chloroform, and centrifuge. This initial extraction diminishes emulsions in the subsequent procedure, and removes any free phenols.

Streptomycin is first removed by adsorption to prepare the "blank urine." In a centrifuge tube mix 5 ml. of urine, containing not more than 1000 γ of streptomycin per ml., with 500 mg. of Lloyd's reagent, measured by means of a calibrated spoon. Shake vigorously and let stand for 15 minutes with occasional shaking. Centrifuge and use the clear supernatant urine in the blank.

1 to 3 ml. of the urine containing a total of 50 to 500 γ of streptomycin (and an equal volume of the blank urine) are diluted to 5 ml. with water

in test-tubes. 1 ml. of 3 N sodium hydroxide is added, and the tubes are immersed in a boiling water bath for 3 minutes and cooled for 3 minutes. The alkaline solution is quantitatively transferred to a 60 ml. separatory funnel by using two rinses with 2 ml. of 1.5 N sulfuric acid. Extract with 10 ml. of washed chloroform for 15 seconds with vigorous shaking. The chloroform layer is transferred to a second funnel and the extraction repeated with another 10 ml. of chloroform. The two chloroform extracts containing maltol are combined and shaken for 15 seconds with 20 ml. of the phosphate buffer, and the chloroform layer is transferred to a third separatory funnel. The buffer solution is washed with 5 ml. of chloroform which is added to the third funnel. Add 5 ml. of water and 1 ml. of 2 N sodium hydroxide to the combined chloroform extracts and shake the funnel for 15 seconds. Most of the chloroform is withdrawn and discarded, and the aqueous phase is transferred to a 15 ml. centrifuge tube with a conical bottom and centrifuged for 2 to 3 minutes. Pipette 5 ml. of the aqueous layer into a test-tube, and add 2 ml. of the 1:1 phenol reagent dropwise. Mix well, let stand 1 to 2 minutes, add 3 ml. of 20 per cent sodium carbonate, and shake vigorously. After 10 minutes the blue color is read in a photoelectric colorimeter at 660 m μ , the solution obtained from the blank urine being used to set the instrument at 100 per cent light transmittance. The instrument corrects for the color caused by interfering substances, and the blue color due to maltol from streptomycin is measured directly.

The extraction procedure should be completed in from 20 to 30 minutes. Protracted contact causes too low results, owing to the influence of chloroform and oxidizing agents. Thus restrict the operation to four funnels; that is, two urines and the corresponding blanks.

Determination in Broth

Reagents

The same as are described under the urine determination

1 N and 7 N NaOH

0.75 and 5 N hydrochloric acid.

Procedure—Most broth samples contain some antifoam agents which are extracted in the procedure and cause the final solution to be cloudy. To remove these substances shake 10 to 20 ml. of the broth for 1 minute in a 40 ml. centrifuge bottle with about an equal volume of washed chloroform and centrifuge for 10 minutes. The clear supernatant is used in all subsequent operations.

1 to 3 ml. of the extracted broth containing a total of 50 to 500 γ of streptomycin is transferred into each of two test-tubes and the volume brought to 4 ml. with water. In the one tube streptomycin is destroyed by adding 1 ml. of 5 N hydrochloric acid and immersing the tube in a boiling

water bath for 5 minutes. After cooling 3 minutes, add 1 ml. of 7 N sodium hydroxide. To the other tube add 2 ml. of 1 N sodium hydroxide and immerse both tubes in a boiling water bath for 3 minutes and cool for 3 minutes. Transfer the contents of each tube quantitatively to 60 ml. separatory funnels, rinsing two times with 2 ml. of 0.75 N hydrochloric acid. The subsequent steps of the extraction are exactly the same as for urine. If only 1 ml. of broth is used for the determination, the washing of the maltol-chloroform layer with the phosphate buffer may be omitted. The acid-pretreated sample is used to set the instrument to 100 per cent transmittance and the untreated broth sample containing streptomycin is read against this setting.

Calibration—The calibration curve is, of course, the same for both urine and broth. Solutions containing from 50 to 500 γ of free base of the

TABLE III
Distribution of Maltol and Alkaline Degradation Products of Streptomycin in Extraction Procedure

Substance	Amount used	Found by direct procedure	Found by extraction procedure; phenol reagent	
			Aqueous phase	Alkaline extract of chloroform phase
	γ	γ	γ	γ
Maltol.....	30	29.7	0.6	31.0
Streptomycin.	147	146	50	104

standard sample are treated in the usual manner for maltol formation and are extracted according to the procedure described. The inactivation of the streptomycin by acid or the adsorption to Lloyd's reagent is unnecessary and the readings are compared to a reagent blank. Beer's law is strictly obeyed.

The calibration curves with the phenol reagent on an aqueous solution of streptomycin directly and after the chloroform extraction are not identical. As was previously pointed out, this is due to an alkaline degradation product of N-methyl-L-glucosamine, which is not soluble in chloroform but does react with the reagent. The figures in Table III clearly show that maltol is completely extracted by the procedure and the same calibration curve applies in this case before or after chloroform extraction. In the case of streptomycin, however, the fraction due to the degradation of the N-methyl-L-glucosamine remains in the aqueous phase and is about 30 per cent of the original color value with the phenol reagent. The sensitivity of the method in which chloroform extraction is employed is therefore about 30 per cent lower than that of the direct method.

DISCUSSION

The *specificity* for the determination of maltol is increased by the chloroform extraction procedure. The alkaline cleavage products of carbohydrates which give the unspecific reactions with the phenol reagent are not soluble in chloroform and are therefore excluded. The reagents used to destroy or adsorb the streptomycin from the blank solutions do not decrease or increase the value of the blank and the readings obtained by difference represent streptomycin. Thus a high degree of specificity results from the restrictions interposed by distribution and by the structural specificity

TABLE IV
Analytical Recovery of Streptomycin from Urine and Broth

	Volume of sample	Streptomycin added	Streptomycin found	Per cent re- covery
	ml	γ	γ	
Urine, human	1	940	950	101
" "	1	470	465	99
" "	1	376	380	101
" "	1	188	184	98
" "	1	141	141	100
" "	1	94	98	104
" "	1	47	50	106
" "	1	268	280	104
" "	2	268	265	99
" "	3	268	275	103
" dog	1	268	275	103
" "	2	536	515	96
Broth	1	148	153	103
"	1	148	135	92
"	1	296	288	97
"	2	148	113	97

of the formation of maltol from streptomycin. This is confirmed by the following observations. In clinical preparations of very low potencies (100 to 250 γ per mg.) occasionally the value obtained by the use of the phenol reagent is higher than that obtained with ferric ammonium sulfate reagent. Furthermore, such samples usually contain larger amounts (up to 50 per cent) of material reacting directly with the phenol reagent. If such samples are assayed by the extraction procedure for broth, values are always obtained which are in excellent agreement with those obtained with the ferric ammonium sulfate reagent.

Recoveries of streptomycin added to both urine and broth were satisfactory and are reported in Table IV.

Reproducibility—None of the points on the calibration curve deviates more than 5 per cent from the best straight line and on the average the deviation is less than ± 3 per cent. The values obtained on samples of urine and broth of unknown streptomycin content can be reproduced to $\pm 10 \gamma$. As little as 10γ of streptomycin per ml. of urine or broth will be detected by the extraction method, provided 3 ml. are used for the test.

TABLE V

Excretion of Streptomycin in Urine of Dogs after Subcutaneous Injection

Dog No.	Streptomycin injected as determined by		Time after injection	Urine volume	Streptomycin in urine as determined by		Streptomycin recovered in urine	
	Chemical assay	Biological assay			Chemical assay	Biological assay	Chemical assay	Biological assay
	γ	γ	hrs.	ml.	γ per ml.	γ per ml.	per cent	per cent
779	89,200	102,600	1	117	245	290	32.1	33.0
			2	120	183	200	24.6	23.4
			3	11	1072	1031	13.2	11.0
			4	105	93	135	10.9	12.8
			5	78	39	49	6.3	5.7
			6	11	192	290	2.3	3.1
Total recovery							89.4	89.0
774	92,900	100,600	1	25	1580	1500	41.5	36.5
			2	86	222	200	20.4	16.9
			3	51	174	150	9.5	7.6
			4	97	75	80	8.3	7.7
			5	70	61	60	4.7	4.1
			7	121	21	24	2.7	2.8
			8	55	7	13	0.4	0.7
Total recovery							87.5	76.3

Both dogs weighed 10 kilos each. Diuresis was induced by administering 200 ml. of water the night before the experiment and at 0, 3, and 6 hours. The stated amount of streptomycin was dissolved in 1 ml. of saline solution for injection.

However, rather large percentage errors are to be expected at these very low concentrations.

The *application* of the streptomycin assay in urine is illustrated in Table V by the protocols on streptomycin excretion in catheter urine of dogs after subcutaneous injection.¹ The per cent deviation of the values ob-

¹ We are indebted to Mr. S. S. Chapman of the Merck Institute for Therapeutic Research for assistance in these experiments and for the microbiological assays on the urine samples.

tained by the chemical and the microbiological assay methods are not recorded, since the microbiological assay in urine is subject to rather wide variations due to other urinary constituents. The two experiments presented in Table V are representative of the fair agreement between the chemical and microbiological assay of streptomycin in urine.

The question whether dihydrostreptomycin is active in the body as such or is converted to streptomycin was decided by the chemical assay in urine. Since dihydrostreptomycin fails to form any maltol on heating with alkali, it cannot be detected by the method based on maltol formation. A

TABLE VI
Assay of Streptomycin in Broth

Microbiological assay	Chemical assay		
	Volume of broth used per test		
	1 ml.	2 ml.	3 ml.
γ per ml	γ per ml.	γ per ml.	γ per ml
125	103	99	110
168	167	166	165
106	127		
98	112	133	
114	120	119	125
132	157	158	152
120	130	136	130
125	126	122	
165	170	185	
73	80		
105	90	108	
173	200	214	
147	152	158	
130	127		
142	154		
126		132	

10 kilo dog was injected intramuscularly with 100,000 units of dihydrostreptomycin dissolved in 2 ml. of saline solution. Urine samples were obtained by catheterization at hourly intervals. The streptomycin determination in the urine was completely negative, although simultaneous microbiological assays showed that the dihydrostreptomycin was excreted as usual. Therefore in the normal dog dihydrostreptomycin is not oxidized to streptomycin.

Table VI gives the results obtained on sixteen broth samples. The chemical values on the average are 9 per cent higher than the biological values. Four of the chemical values were 0 to 10 per cent lower, eight were 0 to 10 per cent higher, and four were 10 to 20 per cent higher than the

corresponding microbiological assays. The extremes of disagreement are larger than in the case of the clinical preparations but are within the reasonable expected range, considering the variation in the microbiological assays. The method has also been used to follow the increase of streptomycin in the nutrient broth during growth of *Streptomyces griseus*, as illustrated in Table VII.

These methods described for urine and broth are not sensitive enough for the assay of streptomycin in blood. A different method with a very high degree of sensitivity will be reported in a separate paper.

TABLE VII
Increase of Streptomycin in Broth during Growth of Streptomyces griseus

Duration of fermentation, hrs.	31	37	43	50
Microbiological assay, γ per ml.... . . .	14	72	135	122
Chemical assay, γ per ml.	18	77	130	115

SUMMARY

A simple and rapid colorimetric method for the assay of streptomycin in clinical preparations, urine, and broth is described. The method is based on the formation of maltol from streptomycin by the action of alkali and the subsequent determination by either the phenol reagent or acid ferric ammonium sulfate.

None of the carbohydrates and amino acids tested interfere under the conditions of the reaction. The formation of maltol is specific for the center portion of the streptomycin molecule, streptose, which must contain an intact carbonyl group and must be glycosidically linked to another group.

The sensitivity of the phenol and ferric reagents is, respectively, 20 to 250 γ and 500 to 2500 γ of streptomycin. The method is reproducible to ± 3 per cent. Satisfactory agreement with microbiological assay values is obtained in clinical preparations.

In the assay of streptomycin in urine and broth, maltol is separated by chloroform extraction from the bulk of the interfering substances prior to the color development. Appropriate blanks are obtained by removal of streptomycin in urine by adsorption to Lloyd's reagent or destruction in broth by boiling with acid. The chemical assay involving extraction is reproducible to ± 10 γ of streptomycin and is in satisfactory agreement with the corresponding microbiological value.

Dihydrostreptomycin is not oxidized to streptomycin in the body of the normal dog.

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16-SUBSTITUTED STEROIDS

III. THE PARTIAL SYNTHESIS OF ESTRIOL

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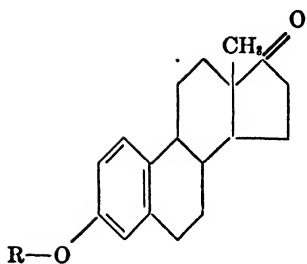
(Received for publication, March 17, 1947)

Estriol was originally obtained (1930) by isolation from human pregnancy urine by Marrian (1) and by Doisy *et al.* (2). Both Doisy (2) and Marrian (3) showed that estriol differed from estrone, another estrogenic hormone occurring in gravid urine, in possessing three acetylatable hydroxyl groups instead of the one hydroxyl and one carbonyl. Butenandt (4) soon (1931) proved that estriol might be regarded empirically as the hydrate of estrone by demonstrating that the trihydroxy estrogen could be dehydrated to produce the ketonic estrogen with the elimination of 1 molecule of water. These findings, therefore, indicated that two of the hydroxyl groups in estriol were vicinal in position and that one of these vicinal hydroxyl groups occupied the same position on the steroid nucleus as that of the carbonyl in estrone. Later researches (5), in which many investigators contributed significant work, served to elucidate fully the correct structural formulas of estrone (I) and estriol (VI).

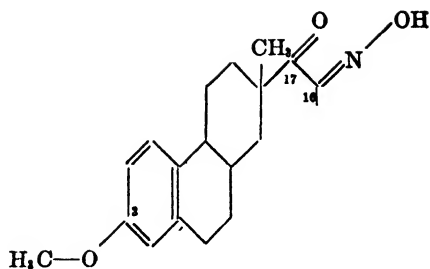
Estriol was first prepared from estrone by the author (6) in 1944. It is now possible to obtain it in over-all yields in the neighborhood of 25 per cent. Exact details for the method are described in this publication. In general, estrone (I) is methylated (7) to produce the 3-methyl ether (II), and the latter is nitrosated (8) to give the 16-oximino derivative (III). The 16-oximino compound is next carried through a Stodola reduction (9), and from this reaction there is obtained a crude α -ketol (IV) which may be either directly submitted to further reduction or else first purified and then reduced to the α -glycol. The reduction to the final carbinol state with pure α -ketol results in a mixture of two isomeric α -glycols; however, from this mixture estriol 3-methyl ether (V) may be easily obtained by fractional crystallization. The synthetic estriol 3-methyl ether is then acetylated, demethylated, and finally saponified to produce estriol (VI) itself.

In this synthesis the salient feature is the use of sodium amalgam in dilute ethanolic acetic acid (temperature 40°) as the agent for transforming the α -ketol to the α -glycol. Reduction of the α -ketol with hydrogen in the presence of a catalyst has always, in our hands, given rise to an isomer (10)

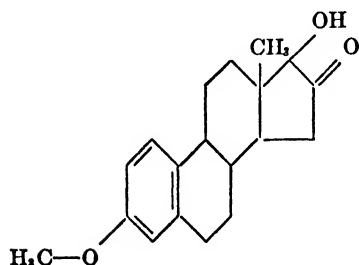
* A portion of the experimentation described in this publication was done at the United States Standard Products Company, Woodworth, Wisconsin.



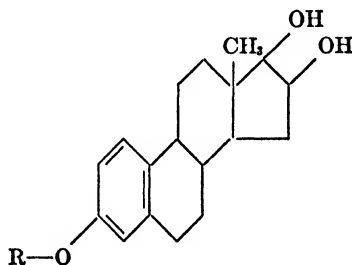
(I) R = H
(II) R = CH₃



(III)



(IV)



(V) R = CH₃
(VI) R = H

of a 16,17-configuration different from that of the naturally occurring estriol.

The transformation of estrone to the 3-methyl ether is essentially a quantitative reaction (11). Nitrosation of this ether to produce 16-oximino-estrone 3-methyl ether was accomplished in 75 per cent yield (11), although Litvan and Robinson (8) report an 89 per cent conversion. Reduction of 16-oximinoestrone 3-methyl ether with zinc and aqueous acetic acid followed by reduction with sodium amalgam (without purification of the intermediate α -ketol) resulted in the production of estriol 3-methyl ether in 46 per cent of the theory. Demethylation was accomplished in 64 per cent yield, giving the total over-all yield from estrone the value of 22 per cent. Calculated on the basis of Litvan and Robinson's figure for the nitrosation, the over-all result would be 26 per cent. There is good reason for believing that this latter figure may be eventually exceeded, as the demethylation process with hydriodic acid was only slightly explored.

EXPERIMENTAL¹

Methylation of Estrone—Estrone 3-methyl ether (II) was prepared as previously described (11).

¹ All melting points reported are uncorrected.

16-Oximinoestrone 3-Methyl Ether (III)—This derivative was prepared in accordance with previous directions (11).

Zinc-Acetic Acid Reduction of 16-Oximinoestrone 3-Methyl Ether—To 279 mg. of 16-oximinoestrone 3-methyl ether (m.p. 178–180°, with decomposition), covered with 10.0 cc. of acetic acid plus 0.6 cc. of water, was added 0.80 gm. of zinc dust, and the resulting mixture was swirled in a bath maintained at 45° until solution of the steroid derivative was complete. Then 9.4 cc. of water were added and the solution refluxed for 1 hour 10 minutes and cooled to room temperature. The aqueous acetic acid solution was decanted from the zinc, and the latter washed with 100 cc. of benzene. To the combined aqueous acetic acid-benzene solutions were added 80 cc. of 1.5 N sodium hydroxide and 200 cc. of ethyl ether. After having been partitioned and separated, the organic phase was washed successively with 0.5 N hydrochloric acid, with 5 per cent sodium bicarbonate, and with water. Evaporation of the ethereal solution yielded a crystalline product which, after treatment with charcoal, was recrystallized once from cyclohexane and once from aqueous ethanol to give 207 mg. of needles melting at 164–165°. Another treatment with charcoal and recrystallization from aqueous acetone raised the melting point to 167–168° (203 mg.). Further recrystallization from aqueous methanol and from aqueous acetone did not alter this melting point.

*Analysis*²— $C_{19}H_{24}O_3$. Calculated. C 75.97, H 8.05

Found. (a) " 75.76, " 8.08

(b) " 75.77, " 7.97

This compound (IV) is undoubtedly identical with the α -ketol (11) (m.p. 162–163°) formed from the titanium trichloride reduction of 16-ketoestrone 3-methyl ether. A mixture of the two ketols gave a sharp melting point of 165–166°. The present α -ketol (m.p. 167–168°) also gave an oxime (11) which melted at 197–198°, with decomposition.

Reduction of α -Ketol Methyl Ether (IV) with Sodium Amalgam—A solution of 800 mg. of α -ketol methyl ether (m.p. 165–166.5°) in 100 cc. of ethanol and 10 cc. of acetic acid was carefully maintained at 40° (water bath), and 200 gm. of freshly prepared sodium amalgam (2 per cent) were added in small pieces with efficient swirling. Before all of the amalgam had been added a precipitation of sodium acetate occurred, and at this point an additional 10 cc. of 50 per cent acetic acid were added. After all the reducing agent had been placed in reaction, the mixture of mercury and solution was transferred to a separatory funnel with ether and water. The mercury plus aqueous phase was separated, after partitioning, from the

² All analyses in this publication were performed by Dr. E. W. D. Huffman, Denver.

ether; the latter was washed with water, with 0.5 N sodium hydroxide, and again with water. Evaporation of the ethereal phase yielded a crystalline residue which was recrystallized twice from acetone-petroleum ether and once from aqueous ethanol to give 431 mg. of tiny leaves (V) melting at 160–163.5°. A subsequent recrystallization from absolute acetone raised the melting point to 161.5–164° (370 mg.).

Acetylation of a small portion of this glycol (m.p. 161.5–164°) gave the diacetate as small flat needles melting at 142.5–143°. Marrian (12) records the melting point of estriol methyl ether as 162.5–164° (corrected) and that of estriol methyl ether diacetate as 140–142° (uncorrected).

Analysis— $C_{23}H_{30}O_6$. Calculated. C 71.48, H 7.83
Found (a) " 71.54, " 7.80
(b) " 71.47, " 7.89

A sample of the synthetic diacetate on admixture with authentic estriol 3-methyl ether-16,17-diacetate (m.p. 140.5–141.5°) showed a melting point of 140.5–141.5°.

Transformation of 16-Oximinoestrone 3-Methyl Ether to Estriol 3-Methyl Ether without Purification of Intermediate α -Ketol—According to the method just described 1.097 gm. of 16-oximinoestrone 3-methyl ether were reduced with zinc and acetic acid (3.0 gm. of zinc dust, 66 cc. of 50 per cent acetic acid, a 1 hour reflux), and the neutral steroid isolated. The yield was 0.926 gm. of crystalline material with a slightly yellow color. Reduction of this crude α -ketolic material with sodium amalgam by the procedure presented above (116 cc. of ethanol, 13 cc. of acetic acid, 250 gm. of 2 per cent sodium amalgam, temperature 40.0–40.5°; finally 10 cc. of 50 per cent acetic acid) yielded 0.488 gm. of estriol 3-methyl ether melting at 158–160° (46 per cent yield).

Demethylation of Estriol 3-Methyl Ether—To a solution of 155 mg. of synthetic estriol 3-methyl ether in 3 cc. of pyridine were added 3 cc. of acetic anhydride; the phases were mixed well and left 48 hours at room temperature. The diacetate was then precipitated by the addition of cold water, and, after having been allowed to stand several hours, filtered, washed well with water, and dried *in vacuo*.

The dry diacetate was then dissolved in 5 cc. of warm acetic acid, 5 cc. of freshly distilled hydriodic acid (sp. gr. 1.7) were added, and the solution of diacetate methyl ether refluxed at once over a free flame. After exactly 5 minutes refluxing 100 cc. of cold aqueous sodium bisulfite were added and the resulting phases mixed well. After precipitation had become complete (ice box), the crystalline steroid was filtered, washed with aqueous sodium bisulfite, and then washed well with water. The residue was dried *in vacuo*.

The demethylated estrogen was then dissolved in 25 cc. of ethanol (room

temperature) and a small spatula end of sodium hydrosulfite added. To the alcoholic solution were next added, for purpose of saponification, 25 cc. of 1 N sodium hydroxide, and the latter was mixed in with rapid swirling. The resulting solution at first became colored a deep yellow but soon turned almost colorless. After 24 hours saponification at room temperature, the ethanolic alkali was added to 250 cc. of 5 per cent sodium bisulfite containing 2.1 cc. of concentrated hydrochloric acid, and the resulting voluminous precipitate extracted with 500 cc. of ethyl ether. The ether, after having been partitioned and separated from the aqueous phase, was washed once with 250 cc. of 5 per cent sodium bisulfite, twice with 200 cc. portions of 0.5 N sodium hydroxide, and twice with water. Evaporation of the ether gave an almost negligible amount of material which was discarded. The combined sodium hydroxide phases (400 cc. of 0.5 N), the bisulfite and water washings having been discarded, were acidified with concentrated hydrochloric acid and extracted with 500 cc. of ethyl ether. This ether, after separation from the aqueous acid phase, was further washed with bicarbonate and with water; a nicely crystalline residue resulted on its evaporation. The crystalline residue (after treatment with charcoal) was recrystallized once from aqueous ethanol to give 62 mg. of estriol (m.p. 266–267°). Recrystallization from acetone-cyclohexane, from aqueous acetone, and from aqueous methanol raised the melting point to 268.5–270°. A mixed melting point performed with authentic estriol (m.p. 268.5–269.5°) showed 269–269.5°.

Analysis— $C_{18}H_{26}O_4$. Calculated. C 74.97, H 8.30

Found. (a) " 74.80, " 8.46

(b) " 74.76, " 8.51

A repetition of this demethylation procedure, in which a larger amount of estriol 3-methyl ether (260 mg.) was employed, gave a better yield of estriol (64 per cent).

SUMMARY

The partial synthesis of estriol is described in detail. From estrone it is possible to prepare estriol 3-methyl ether in 35 to 41 per cent yield, and estriol itself in 22 to 26 per cent over-all yield.

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MICROGASOMETRIC ESTIMATION OF THE BLOOD GASES

V. COMBINED CARBON DIOXIDE AND OXYGEN

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The determination of carbon dioxide in small (12 c mm.) samples of blood by the syringe method requires vacuum extraction (1). This technique has been improved by Lilienthal and Riley (2) and by technical developments in this laboratory, so that the carbon dioxide determination is now quite simple to perform. With slight amplification of the technique it is also now possible to carry out determinations of carbon dioxide and oxygen in a single sample of blood. The combined method can be learned readily and offers a considerable increase in the range of usefulness of the syringe analyzer, inasmuch as it also permits the analysis of blood from birds, reptiles, amphibians, and fishes, which hitherto has been extremely difficult or impossible to handle with the syringe analyzer.

The accuracy of the combined analysis, as controlled against analyses by the method of Van Slyke and Neill (3), is ± 1 cc. of gas in 100 cc. of blood. The accuracy of oxygen determination in mammalian blood is even better, but cannot equal the accuracy of determination of oxygen alone in the larger samples used in the simple method for oxygen determination described by Roughton and Scholander (4).

Apparatus—

1. The *syringe analyzer* is that described by Scholander and Roughton (5). The syringe has been modified to make it easy to hold the plunger out during the vacuum extraction by providing a notch in the plunger which engages a spring steel clip. To cut the notch draw the plunger out 0.8 cc., and mark it 3 to 4 mm. past the syringe barrel. Cut the notch in the plunger with a triangular medium or fine India oil stone straight below the number of the plunger, slanting the stone so that the spring clip will not disengage the notch during vacuum extraction.

2. *Plastic-tipped wire plug* (Fig. 1, A), to seal the capillary during vacuum extraction. The tip is fashioned from a piece of 1.5 to 2 mm. plastic cord¹ by rotating and gently heating it in front of a micro flame, and pulling it apart when the plastic becomes soft. The pointed tip so formed is cut

¹Transflex rodding, Irvington Varnish and Insulator Company, Irvington 11, New Jersey.

off to a length of 2 to 3 mm., pierced straight with a gage No. 24 syringe needle, and threaded onto a $\frac{3}{4}$ to 1 mm. steel wire which is bent as illustrated. A rubber band attaches the wire plug to the syringe barrel, as shown in Fig. 1.

3. *Wire clip* (Fig. 1, B), to give the plunger a suitable friction and to hold the plunger out during vacuum extraction. A 6 to 7 mm. wide ring is cut off from a piece of thick walled rubber tubing and is slipped firmly onto the syringe. A straight angle bow of 0.4 to 0.5 mm. of music

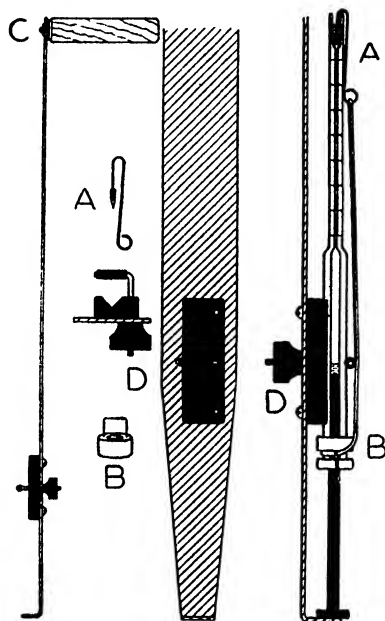


FIG. 1. Capillary syringe and sling for the combined CO_2 and O_2 method. A, plastic-tipped wire plug; B, wire clip stuck in section of rubber tubing; C, sling handle; D, cradle for fastening the syringe analyzer.

wire, wide enough to slip over the syringe collar, is stuck straight through the rubber tubing. With the clip engaged in the plunger notch, the end of the plunger should read 80 (0.8 cc.).

4. *Hand sling* for the syringe, for whirling down liquid bridges in the capillary and for breaking up bubbles in the syringe. The sling is 4 cm. wide by 50 cm. long, made from 1.5 mm. of sheet aluminum. It can be whirled around a handle (Fig. 1, C). The other end is bent out at a straight angle. It is furnished with a plastic cradle (Fig. 1, D) which carries a screw hook padded with rubber tubing for fastening the syringe, as seen in Fig. 1.

Blood Sample—For the general technique in handling the blood see the paper on oxygen determination by the syringe technique (5). If the blood supply is very scant, it may be necessary to handle samples in the smallest possible syringe with a minimum amount of dead space for the heparin-fluoride solution. Such a syringe can easily be prepared from a 0.25 cc. tuberculin syringe which is provided with a wire brake applied to the plunger as described (Fig. 1, *B*), to give proper friction to the plunger. The nozzle of the syringe is preferably ground down straight on a piece of emery paper to a 1 or 2 mm short stub and the capillary opening is ground slightly funnel-shaped by means of a conical steel rod and a little emery powder. It will then fit the pipette tip perfectly. A short syringe needle of the correct gage to fit snugly into the bore of the nozzle is broken near its nipple, ground square at the broken end, and stuck into the syringe nozzle, where it is sealed in position with a drop of beeswax.

After the blood is drawn into the syringe, it is followed by a drop of mercury to be used for mixing the blood. The needle is detached from the nozzle, the wax is scraped off, and the blood can now be delivered directly into the blood pipette. Between analyses the blood syringe is sealed off with a drop of hot wax on the cut off nozzle and is stored in ice water.

The blood pipette can be the pipette described in the first report on the syringe analyzer (4), with ground tip, or any similar smaller pipette. The tip must be ground conical so as to fit the cup of the syringe analyzer. This is easily done by rotating it with wet fine carborundum powder in a small conical depression drilled or punched in a metal plate. As the blood is measured in the graduated capillary of the syringe analyzer, the pipette needs no calibration.

Principle of Method—Ferricyanide solution and caprylic alcohol are pre-extracted in the syringe to remove dissolved gases. The blood sample is laked in the ferricyanide in the syringe, driving oxygen from the chemical combination into physical solution. Acid buffer is then added to release the CO_2 . The solution is vacuum-extracted in the syringe by closing the capillary with a plastic-tipped wire plug and pulling the plunger out to a position fixed by a spring clip engaging a notch in the plunger. The syringe is whirled in the hand sling to clear the capillary from liquid bridges and to break up bubbles in the blood solution. The vacuum is released by letting in the plunger, and the volumes of gas are measured in the graduated capillary before and after each successive absorption with alkali and alkaline pyrogallol. The differences between measurements (less blanks to correct for air bubbles introduced and dissolved gases in the reagents), multiplied by an adjustment factor to standard temperature and pressure, give the cc. of CO_2 and O_2 in 100 cc. of blood. Reabsorption of CO_2 during the compression is checked by using an acid

buffer of low CO_2 solubility, by eliminating bubbles in the syringe, and by providing a capillary well drained by centrifugation for the gas to escape into while it is still at a low pressure.

Reagents—

1. Glycerol-aquaresin mixture,² made by mixing equal parts of the two reagents. This is stored in a 2 cc. syringe with a No. 20 needle, the tip of which has been ground off square.

2. Ferricyanide solution. 1 gm. of potassium ferricyanide, $\text{K}_3\text{Fe}(\text{CN})_6$, and 0.25 gm. of saponin are dissolved in 10 cc. of distilled water, and 0.1 cc. of acid-phosphate buffer is added to the solution.

3. Caprylic alcohol.

4. Acid-phosphate buffer. 95 gm. of acid-sodium phosphate, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, are dissolved in 100 cc. of distilled water to make an almost saturated solution.

5. Distilled water.

6. 10 per cent NaOH .

7. Pyrogallol solution. 15 gm. of powdered pyrogallol are added to 100 cc. of 20 per cent NaOH in a rubber-stoppered bottle, and the solution is covered with a layer of oil 2 cm. thick. The pyrogallol is dissolved by stirring with a glass rod passing through the oil.

Reagents 2 to 7 are kept for current use in 2 or 5 cc. syringes with glass tips attached through rubber tubing.

Procedure

For CO_2 and O_2 —1. Remove the plunger from the syringe, rinse it well with hot water, and dry by suction. This is easily done by attaching a small glass tube to the vacuum line and inserting this tube to the bottom of the barrel, so that air is pulled through the warm capillary and barrel.

2. Introduce a drop of glycerol-aquaresin into the barrel opening. Lubricate the dry plunger with a few streaks of the glycerol solution and return it to the barrel, adding enough of the solution so that no air is carried in with the plunger. Unless the plunger is moved gently in the barrel, the latter may break because of the viscosity of the glycerol solution. When the plunger strikes the bottom, the glycerol solution should rise only a few mm. in the capillary.

3. Fill the cup with ferricyanide, draw down into the barrel, and expel it without trapping air bubbles. Remove the excess, leaving only the dead space filled with ferricyanide.

4. Place a drop of caprylic alcohol on top of the ferricyanide. Draw down 2 scale divisions of the alcohol; suck out the remainder. Draw

² Aquaresin is obtainable from the Glyco Products Company, Inc., New York.

out the plunger until the fluid meniscus is lowered 1 to 2 mm. into the capillary.

5. Seat the plastic-tipped wire plug in the cup, trapping a small air bubble on top of the caprylic alcohol. The rubber band must be strong enough to keep the capillary firmly closed by the plastic tip. Add a little ferricyanide in the cup to complete the seal.

6. Pull the plunger out slowly until the spring clip engages the notch in the plunger. Add glycerol-aquaresin solution around the plunger bearing. If the caprylic alcohol stays in the capillary, it may be moved down into the barrel by warming the capillary behind it in the hand. Allow a few seconds for extraction and release the vacuum by letting the plunger in. Expel the bubble and repeat the extraction.

7. Lower the meniscus of the extracted fluid 1 to 2 mm. into the capillary. Place the tip of the blood pipette firmly against the bottom of the cup, trapping a small (0.5 to 1 division) air bubble between the blood and the extracted fluid. Draw the blood slowly in until the length (A_1) of the air bubble can be measured. Continue to draw blood in very slowly and evenly until 28 to 29 divisions have entered. Remove the blood pipette, suck off excess blood, adjust the upper blood meniscus to the zero mark, and read the volume of the blood sample: b divisions. With practice the blood volume can be made very close to 30 divisions.

8. Fill the cup to the mark with acid-phosphate solution, leaving a small (0.5 to 1 division) air bubble between the buffer and the blood. Draw slowly in until the length (A_2) of the air bubble can be measured. Turn the syringe cup down and continue to draw in phosphate buffer slowly and evenly until the bubble reaches the junction of the capillary with the syringe barrel, preventing the acid from entering the syringe barrel.

9. Rotate the syringe on an oblique axis, cup down, until the blood is completely laked to a uniform dark brown color by the ferricyanide-saponin solution. If more than a trace of acid buffer enters the blood ferricyanide-saponin solution, the blood will not lake properly and the oxygen will not be completely released from the hemoglobin.

10. Hold the syringe cup up. Draw in the remainder of the acid-phosphate solution and close the capillary with the plastic-tipped wire plug, trapping a small air bubble (0.5 division or less) underneath. Estimate the length of this bubble (A_3). Add a drop of acid buffer to the cup to complete the vacuum seal.

11. Pull the plunger out slowly so that the capillary drains properly when the small bubble underneath the plug expands. Continue until the plunger notch engages the clip. Add the glycerol solution around the plunger bearing

12. Attach the syringe to the sling as illustrated. It is necessary that the plunger head should rest against the bent end of the sling. Whirl the blood solution down to the bottom of the syringe and tilt back and forth several times to have the liquids well mixed. Whirl the sling again for about 30 seconds. The capillary should be clear of bridges and the blood solution free of bubbles. Avian, reptilian, amphibian, and fish blood make a very viscous solution, which may make it impossible to get rid of bubbles completely. If the volumes of these vacuum bubbles are insignificant compared with the total vacuum space in the syringe, they will not affect the result.

13. Remove the syringe from the sling without touching the capillary. Hold the cup up. Release the plunger stop and move the fluid quickly up into the capillary until atmospheric pressure is almost reached.

14. Without delay remove the plug and adjust the upper gas meniscus to the zero mark and read the volume (V_1) of the gas bubble. Since the capillary has not been handled at any time, there is no necessity for temperature equilibration in a water bath. Delay and fumbling in Steps 13 and 14 result in some reabsorption of CO_2 .

15. Fill the cup with water. With the syringe held cup up pull most of the water down into the syringe, where it will form a layer on top of the heavy blood solution. Immediately return the bubble to the capillary, leaving water behind it on both sides of the gas in the capillary. Owing to absorption of CO_2 by this washing the bubble is now much shorter.

16. Remove the excess water; fill the cup with NaOH . Invert the syringe, run the gas bubble out into the alkali, and rotate a few times to facilitate absorption. Draw some alkali into the capillary and then the gas bubble. Measure its volume (V_2).

17. Remove NaOH from the cup and fill with pyrogallol solution. Run the bubble out into the cup and absorb as in the previous step. Return the bubble to the capillary and read the volume; record and designate it V_3 in calculations.

18. Remove the plunger gently from the barrel under the water faucet. Shake out the contents of the barrel and wash with running tap water several times before the plunger is again inserted. It is recommended that dichromate cleaning solution be used after each analysis to insure that no protein remains sticking to the capillary. While the syringe is still hot, dry it by suction, as described in Step 1.

For CO_2 —If carbon dioxide alone is desired, follow Steps 1 to 3, but substitute for ferrieyanide water freed from CO_2 by boiling with a drop of H_2SO_4 . In Step 4 omit the caprylic alcohol. Proceed to Step 7, which is followed through, omitting the measurement of the air bubble which separates the blood from the acidulated water. Add 2 divisions

of caprylic alcohol on top of the blood, and immediately on top of the alcohol a cup full of acid-phosphate buffer. Draw in the acid and close the capillary, as in Step 10. Follow Steps 11 through 16. Calculations are as for the combined method.

Calculations

CO_2 -Volumes per cent of CO_2 in blood = $(V_1 - V_2 - c_1) \times f \times 100/b \times i$. c_1 is the blank correction for the CO_2 content of the reagents. It is determined by running an analysis with all the reagents, but no blood. In our hands it is 0.1 capillary division. f is the factor for adjusting gas volumes from the observed temperature, barometric pressure, and water vapor to their dry volumes at 0° and 760 mm. (see Table I). b is the blood volume in terms of divisions of the capillary. i is the combined correction factor for the incomplete extraction of CO_2 and for the CO_2

TABLE I
Factors for Reducing Volume of Moist Gas to Volume Occupied by Dry Gas
at 0° and 760 Mm. of Hg*

Hg	16°	18°	20°	22°	24°	26°
750	0.91	0.90	0.90	0.89	0.88	0.87
755	0.92	0.91	0.90	0.89	0.88	0.87
760	0.93	0.92	0.91	0.90	0.89	0.88
765	0.93	0.92	0.91	0.90	0.89	0.89

* Abbreviated from Peters and Van Slyke (6) (Table XV, p 129).

reabsorbed by the solutions. It was found to average 1.015, which is the same figure as that determined in the syringe method for CO_2 in blood (1).

O_2 -Volumes per cent of O_2 in blood = $(V_2 - V_3 - c_2 - a) \times f \times 100/b \times i$. c_2 is the blank correction for the O_2 contents of the reagents used. In our hands it is 0.2 capillary division. a is the O_2 content of the air bubbles introduced during analysis in order to separate reagents. $a = 21$ per cent ($A_1 + A_2 + A_3$). f is the factor for the adjustment of moist gas at observed temperature and barometric pressure to the dry condition at 0° and 760 mm. (see Table I). i is the combined correction factor for the incomplete extraction of O_2 and for the reabsorption of O_2 by the solutions. It was found to average 1.011. The i factor was obtained as the average of the factors by which the results with the syringe (in other respects corrected) must be multiplied to equal the results of the Van Slyke analysis.

Accuracy of Results—Estimations of the CO_2 and O_2 content of blood by the combined method were checked against determinations by the

Van Slyke and Neill manometric method (3). The results of a consecutive series of determinations on mammalian blood samples are given in Table II.

TABLE II
CO₂ and O₂ Content of Mammalian Blood Determined by Syringe and by Van Slyke Method

The values are given as volumes of gas in 100 volumes of blood.

	CO ₂ , syringe, 12 c mm.	CO ₂ , Van Slyke, 1 cc.	O ₂ , syringe, 12 c mm.	O ₂ , Van Slyke, 1 cc.
Venous blood, man	55.3, 56.6	57.4, 57.5	6.4, 6.4	5.6, 5.6
	56.7, 56.3	57.7	5.8, 6.4	5.7
	57.0, 56.7		6.1, 5.8	
Aerated " "	29.7, 29.3	29.4, 29.3	22.4, 22.6	22.2, 22.3
	29.6, 31.1	29.7	22.6, 21.4	21.8, 21.9
	30.5, 31.1		22.5, 22.0	
	45.1, 45.3	45.3, 45.4	12.0, 11.8	12.6, 12.3
Arterial blood, rabbit	45.4, 45.3		13.0, 12.1	
	45.1, 43.7		12.5, 12.4	
	45.4		12.1	

TABLE III
CO₂ and O₂ Content of Avian and Fish Blood Determined by Syringe and by Van Slyke Method

The values are given in volumes per cent.

	CO ₂ , syringe, 12 c mm.	CO ₂ , Van Slyke, 1 cc.	O ₂ , syringe, 12 c mm.	O ₂ , Van Slyke, 1 cc.
Venous blood, duck	37.9, 39.7	39.7, 39.5	15.5, 15.5	15.6, 15.4
	39.4, 39.9	39.3	14.3, 15.6	16.3
Aerated " "	20.3, 20.3	20.7, 21.1	19.2, 19.7	20.6, 20.2
	21.1, 20.5	21.1	19.5, 16.9	19.7
	20.8		19.0	
Heart " carp	29.4, 29.6	29.9, 30.5	5.2, 4.9	5.7, 5.4
	27.7, 27.8		4.9, 5.8	
	29.7, 30.5		5.2, 4.9	
	30.1		4.5	
Aerated " "	19.9, 18.2	18.4, 18.6	11.0, 10.8	12.0, 12.0
	19.3, 20.0		11.0, 11.1	
	18.4, 18.0		11.0, 11.5	
	18.7		11.1	

Out of nineteen consecutive carbon dioxide determinations in mammalian blood by the syringe method fifteen are within ± 1 per cent of the standard value.

Out of nineteen consecutive oxygen determinations in mammalian blood fifteen are within ± 0.6 per cent of the standard value, and all the results are within ± 0.7 per cent.

The combined method was also applied to the determination of CO_2 and O_2 in the blood of birds and fishes. The results of a consecutive series of determinations are given in Table III.

Out of twenty-three carbon dioxide determinations in avian and fish blood by the syringe method nineteen came within ± 1 per cent of the standard value.

Out of twenty-three oxygen determinations in avian and fish blood twenty came within ± 1 per cent of the standard value.

SUMMARY

The syringe analyzer method of Scholander and Roughton is adapted for the determination of the CO_2 and O_2 content of a single sample of blood. O_2 is released from chemical combination by ferricyanide and CO_2 is evolved by the addition of acid-phosphate buffer. The gases are vacuum-extracted in the syringe, selectively absorbed, and measured in the capillary. The volume of blood required for a determination is 12 c.mm. The accuracy for both gases is within ± 1 per cent for mammalian, bird, and fish blood. The time necessary for an analysis is 15 to 20 minutes.

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THE SYNTHESIS OF PHOSPHOMALIC ACID*

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Esterification of inorganic phosphate coupled to the oxidation of fumarate or malate in tissue suspensions has been repeatedly demonstrated (1-4). Kalckar (2) and Leloir and Muñoz (4) have shown that during the oxidation of malate or fumarate a phosphate ester having the properties of phosphopyruvic acid is formed. In explanation of these data, Lipmann (5) has suggested that fumarate combines with inorganic phosphate to form an intermediate phosphorylated ester, namely phosphomalic acid, which is then postulated to undergo oxidation to phosphoenoloxalacetate. Decarboxylation of the latter would yield phosphopyruvate. Solomon *et al.* (6), have concluded that such a mechanism for phosphopyruvate synthesis would account for observed isotope distribution in their study of glycogen formation from lactate. The esterification of phosphate during malate or fumarate oxidation may also have considerable significance in view of the fact that oxalacetate can act as an intermediate hydrogen acceptor for as many as 8 of the 12 hydrogen atoms released in the oxidation of 1 molecule of triose phosphate (7).

In view of the general importance of these suggestions it appeared that study of the esterification mechanism might be aided if the hypothetical intermediate were available. This paper deals with the synthesis and properties of the optical isomers of phosphomalic acid, the intermediate postulated by Lipmann.

Several attempts to phosphorylate malic acid with phosphorus oxychloride by the method used by Wagner-Jauregg (8) in the preparation of phospholactic acid failed. This approach was abandoned when it became evident that the main product of the reaction was inorganic pyrophosphate. An attempt to phosphorylate malic acid directly with diphenylphosphoryl chloride by the method of Brigl and Müller (9) was also unsuccessful, the main product of the reaction being diphenylphosphoric acid. However, it was found that diethyl malate in pyridine could be readily phosphorylated with diphenylphosphoryl chloride at room temperature. The phenyl groups were then removed by hydrogenolysis in the presence of platinum oxide catalyst, and the resulting diethyl ester of phosphomalic

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† National Institute of Health Research Fellow.

acid was saponified by dilute alkali at 38°. The barium salt of phosphomalic acid was precipitated from an alkaline medium, freed of inorganic phosphate with magnesia mixture, and purified by reprecipitation. The over-all yield was approximately 50 per cent of theory.

Barium phosphomalate is only slightly soluble in water but quite soluble in acid. The ratio of barium to phosphomalic acid in the precipitated salt depends on the conditions of precipitation. Precipitation of the barium salt from a distinctly acid solution by addition of 6 volumes of ethanol yields a salt containing 1.3 moles of barium per mole of phosphorus, whereas precipitation at pH 8.5 without addition of alcohol yields a compound with a Ba:P ratio of 1.8 to 1.9. A ratio of 1.96 is attained by precipitation of the barium salt at pH 10. Other analytical data show that the Ba:P ratio is an indication of the relative amounts of monobarium and dibarium phosphomalate present in the salt.

Crystalline brucine salts of *d*- and *dl*-phosphomalic acid were obtained. Insoluble lead and silver salts were also prepared but were not investigated further.

Phosphomalic acid is fairly stable to 1 N H₂SO₄ at 100° and to alkali at room temperature. It is readily hydrolyzed by the acid phosphatase of human seminal fluid

EXPERIMENTAL

Diphenylphosphoryl Chloride--Diphenylphosphoryl chloride was prepared by the method of Brigl and Müller (9) and redistilled at 209–212° (21 to 25 mm.).

Diethyl l-Malate—*l*-Malic acid was esterified by the method of Fischer and Speier (10). The rotation of the ester was $[\alpha]_D^{24} = -15.9^\circ$ (0.6304 gm. in 10.00 ml. of acetone) as compared with a value of -14.16° reported by Walden (11).

Diethyl-l-diphenyl Phosphomalate—To 16.62 gm. of diphenylphosphoryl chloride in 11 ml. of dry pyridine were added 11.75 gm. of diethyl *l*-malate in 6 ml. of pyridine. Crystals of pyridine hydrochloride appeared immediately in the reaction mixture. Although heat was evolved, cooling was not necessary. 9 ml. of pyridine were used as a wash to complete the addition of diethyl malate. The reaction mixture was allowed to stand at room temperature for 28 hours. Water, 100 ml., was then added and the pH was adjusted to 8.0 by the addition of 7.5 ml. of 9.8 N NaOH. The two phase mixture was extracted with three portions of ether (total volume, 200 ml.). The ether extract was washed with four portions of 0.5 N H₂SO₄ (total volume, 200 ml.), and three portions of water (total volume, 150 ml.). The washed ether extract was then taken to dryness *in vacuo*. Ethanol was added after evaporation of the ether and the solu-

tion was again taken to dryness. After several such additions of ethanol, the oil residue was dried in a vacuum oven at room temperature for 20 hours. The yield was 22.46 gm., or 86 per cent of theory. The rotation was $[\alpha]_D^{24} = -6.49^\circ$ (0.5931 gm. in 5.00 ml. of acetone).

Analysis— $C_{20}H_{22}O_3P$. Calculated, P 7.35; found, P 7.31, 7.38

Diethyl l-Phosphomalate—Hydrogenolysis of diethyl *l*-diphenyl phosphomalate was accomplished by the method of Brigl and Müller (9). 1.6746 gm. of diethyl *l*-diphenyl phosphomalate in 50 ml. of ethanol were hydrogenated at room temperature and atmospheric pressure in the presence of 155 mg. of platinum oxide catalyst. At the end of 4 hours the uptake of hydrogen had completely ceased. The uptake was approximately 860 ml. as compared to a calculated uptake of 840 ml. The ethanol solution was filtered and evaporated *in vacuo*. The oil residue was taken up with a small amount of ethanol and neutralized with alkali, requiring 75.9 ml. of 0.1 N NaOH, or 95.5 per cent of theory. Water was added to 100 ml.

Saponification of Diethyl l-Phosphomalate—To a 10 ml. aliquot of diethyl disodium *l*-phosphomalate, representing the hydrogenolysis product of 0.1675 gm. of diethyl *l*-diphenyl phosphomalate, were added 1.0 gm. of barium acetate in 5 ml. of water and 10 ml. of 0.1 N NaOH. The barium salt of phosphomalate gradually precipitated as the saponification proceeded. The mixture was allowed to stand overnight at room temperature. It was then adjusted to neutrality by the addition of 2.18 ml. of 0.1 N HCl. Thus 0.78 milliequivalent of alkali was used for the saponification of the diethyl ester, or 99 per cent of theory. A separate 25 ml. aliquot showed an uptake of 2.04 milliequivalents of alkali, or 101 per cent of theory. This method of saponification was therefore quantitative and was employed in the preparation of larger batches of barium *l*-phosphomalate.

Barium l-Phosphomalate—The hydrogenolysis product of 2.01 gm. of diethyl *l*-diphenyl phosphomalate was neutralized with 97.3 ml. of 0.1 N NaOH. To the solution were added 2.7 gm. of barium acetate and 120 ml. of 0.1 N NaOH. The solution was allowed to stand overnight at 38°. The barium precipitate was combined with that of a duplicate batch and centrifuged. The barium salt was dissolved in 125 ml. of water with the addition of 2.8 ml. of 12 N HCl. The barium salt was then precipitated by adding 10 N NaOH to pH 8.5. After centrifugation the barium salt was redissolved and reprecipitated two more times as described above. It was finally washed by three resuspensions in 50 ml. portions of water. After two suspensions in absolute alcohol, the barium salt was centrifuged and dried *in vacuo* for 24 hours at 60°. The weight of the salt was 3.51 gm., a yield of approximately 70 per cent of theory.

Purification of Barium *l*-Phosphomalate—To 2.7 gm. of powdered barium *l*-phosphomalate were added 43 ml. of 0.5 N H_2SO_4 . The suspension was shaken for 20 minutes. The barium sulfate was centrifuged and washed once with 10 ml. of water. The first supernatant layer and the wash water were combined. 1 ml. of 12 N HCl and 0.8 gm. of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in 10 ml. of water were added. 15 N ammonia was then added to pH 8.5. The solution was placed in a refrigerator for 4 hours. It was then filtered through a cotton-asbestos disk. The filtrate was adjusted to pH 2.8 by addition of 12 N HCl. Barium acetate was added to rid the solution of sulfate. After centrifugation and filtration through cotton-asbestos, the clear filtrate was treated with 4.0 gm. of barium acetate. The precipitate which appeared at this point was put into solution by further addition of 12 N HCl. The solution was now completely clear. 15 N ammonia was then added to pH 8.5. The barium salt was centrifuged and washed twice by suspension in 40 ml. portions of dilute ammonia. The salt was dried *in vacuo* for 25 hours at 60°. The yield was 2.245 gm. or approximately 83 per cent recovery. The rotation was $[\alpha]_D^{25} = -4.03^\circ$ (0.2729 gm. in 5.00 ml. of 2.2 N HCl). The specific rotation of *l*-phosphomalic acid based on the quantity of esterified phosphorus present in the barium salt was -9.7° .

The salt was found to be a mixture of 83.7 per cent dibarium phosphomalate, 7.9 per cent monobarium phosphomalate, 8.1 per cent water, and 0.3 per cent barium phosphate. The Ba:P ratio was 1.89. (Ba is corrected for the amount equivalent to inorganic P in all ratios given in this paper. The P value used is esterified P.)

Analysis—Calculated. C 9.39, H 1.53, Ba 50.8, P 6.08, inorganic P 0.03
 Found. " 9.71, " 1.45, " 50.8, " 6.08, " " 0.03

Barium *dl*-Phosphomalate—Racemic barium phosphomalate was prepared by methods described in this paper with two modifications to ascertain best conditions for precipitation of the dibarium salt.

1. Crude barium phosphomalate, prepared by the hydrogenolysis and subsequent saponification of 6.81 gm. of diethyl diphenyl phosphomalate, was suspended in 200 ml. of water and dissolved by addition of 12 N HCl. 6 volumes of ethanol were then added. The precipitate was centrifuged and dried in a vacuum oven at 60°. The Ba:P ratio was 1.31.

Analysis—Found, Ba 41.7, P 7.19, inorganic P 0.11

2. The alcohol-precipitated barium salt was dissolved in HCl, barium acetate added, and the salt reprecipitated by the addition of 15 N ammonia to pH 8.5. The suspension was allowed to stand for 1 hour before centrifugation. After two washings with dilute ammonia and final suspension in

ethanol, the barium salt was centrifuged and dried at 60° *in vacuo*. The Ba:P ratio was 1.96.

Analysis—Found, Ba 53.5, P 6.17, inorganic P 0.13

Inspection of the above analytical data shows that precipitation of dibarium phosphomalate is dependent on adjusting the pH to 8.5. A lower pH favors the precipitation of monobarium phosphomalate.

d-Malic Acid—The method of Dakin (12) was used for the resolution of *dl*-malic acid.

Diethyl d-Malate—The diethyl ester of *d*-malic acid was prepared according to the method of Fischer and Speier (10) as described for the preparation of diethyl *l*-malate. The ester boiled at 125–127° (10 mm.). The rotation was $[\alpha]_D^{25} = +16.5^\circ$ (0.6577 gm. in 10.00 ml. of acetone). This rotation compares favorably with the opposite rotation of -15.9° obtained for diethyl *l*-malate.

Barium d-Phosphomalate—The synthesis of diethyl *d*-phosphomalate was accomplished as described for the *l* isomer. The hydrogenolysis product of 7.17 gm. of diethyl *d*-diphenyl phosphomalate (three separate hydrogenations of 2.39 gm. each) was saponified by treating the oil with 750 ml. of 0.1 N NaOH for 22 hours at 38°. The solution was then acidified with 9 ml. of 12 N HCl. 2 gm. of $MgCl_2 \cdot 6H_2O$ in 4.0 ml. of water were added and 15 N ammonia to pH 8.5. The solution was placed in a refrigerator for 2 hours and then filtered through two filter disks. The filtrate was treated with 9 gm. of barium acetate. 12 N HCl was added until the barium precipitate dissolved. The flask was then evacuated to remove CO_2 . 15 N ammonia was added to pH 8.5. The barium salt was centrifuged and washed twice by suspension in 50 ml. portions of dilute ammonia. After a final suspension in 100 ml. of ethanol, the barium salt was centrifuged and dried *in vacuo* at 60° for 24 hours. The yield was 5.58 gm., or 63 per cent of theory. The rotation was $[\alpha]_D^{25} = +4.08^\circ$ (0.2430 gm. in 4.50 ml. of 2.2 N HCl). The specific rotation of *d*-phosphomalic acid, based on the quantity of esterified phosphorus in the barium salt, is $+9.7^\circ$. This rotation agrees with the opposite rotation of -9.7° given by *l*-phosphomalate and indicates strongly that no racemization took place in the synthetic procedures. The Ba:P ratio was 1.79.

Analysis—Found, Ba 50.2, P 6.40, inorganic P 0.32

Purification of Barium d-Phosphomalate—The following is the preferred procedure for the purification of phosphomalic acid, since it yields the dibarium salt free of all but traces of inorganic phosphate. To 2.55 gm. of powdered barium *d*-phosphomalate were added 37.27 ml. of 0.5 N H_2SO_4 .

The suspension was shaken for 45 minutes and then centrifuged. 1 ml. of 12 N HCl and 1.0 gm. of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in 5 ml. of water were added to the supernatant liquid. 15 N ammonia was added to pH 10. The solution was allowed to stand in the refrigerator overnight and then filtered through a cotton-asbestos pad. 4 drops of 2 M barium acetate were added to free the solution of any sulfate present. After filtration, the solution was poured into 9.3 ml. of 2 M barium acetate (100 per cent excess) with vigorous stirring. After 1 hour the barium salt was centrifuged and washed with 100 ml. portions of the following: 1 per cent barium acetate (adjusted to pH 10 with ammonia and filtered), 50 per cent ethanol plus 2 drops of 15 N ammonia, 75 per cent ethanol plus 2 drops of 15 N ammonia, 95 per cent ethanol, and ether. The salt was dried *in vacuo* at 60° for 16 hours. The yield was 2.08 gm., or a recovery of 81 per cent. The Ba:P ratio was 1.96.

Analysis—Found, Ba 52.5, P 6.08, inorganic P 0.07

Tetrabrucine Salt of dl-Phosphomalate—To 250 mg. of barium *dl*-phosphomalate were added 4 ml. of water and 4.0 ml. of 0.5 N H_2SO_4 . The suspension was shaken for 15 minutes. The barium sulfate was centrifuged off, and the clear supernatant neutralized by addition of KOH. 7 ml. of saturated aqueous brucine hydrochloride were added. A cottony mass of fine long needles was obtained after 2 days at 5°. The substance was recrystallized twice from hot water and dried *in vacuo* at 60°. The yield was 223 mg., or 24 per cent of theory. The crystals sintered with decomposition at 142–148°. Analytical values corresponded to those of a salt containing 4 moles of brucine per mole of phosphomalic acid.

Analysis— $\text{C}_{96}\text{H}_{111}\text{O}_{24}\text{N}_8\text{P} \cdot 5\text{H}_2\text{O}$

Calculated. C 61.25, H 6.48, N 5.96, P 1.65

Found. " 61.23, " 6.56 " 5.93, " 1.64

Brucine d-Phosphomalate—500 mg. of barium *d*-phosphomalate were dissolved in 2.5 ml. of water and 0.35 ml. of 12 N HCl. 10 ml. of saturated aqueous brucine hydrochloride were added and soon thereafter short blunt needles appeared. The brucine salt, recrystallized three times from water and dried *in vacuo* at 60°, weighed 306 mg., a yield of 50 per cent of theory. The crystals sintered with decomposition at 149–154°. The analytical values corresponded to those of a compound containing 1 mole of brucine per mole of phosphomalic acid.

Analysis— $\text{C}_{27}\text{H}_{31}\text{O}_{11}\text{N}_2\text{P} \cdot \frac{1}{2}\text{H}_2\text{O}$

Calculated. C 52.52, H 5.55, N 4.54, P 5.02

Found. " 52.52, " 5.64, " 4.53, " 5.01

Stability of Phosphomalic Acid to Acid and Base—Phosphomalic acid is quite resistant to acid hydrolysis, as indicated by the data in Table I. After 3 hours in 1 *N* H₂SO₄ at 100°, 19 per cent of the esterified phosphorus is hydrolyzed. No appearance of inorganic phosphorus was detected when phosphomalic acid was treated with either 1 *N* NaOH for 20 minutes at 20° or alkaline iodine.

Action of Acid Phosphatase on Phosphomalic Acid—Phosphomalic acid was readily dephosphorylated by a preparation of acid phosphatase obtained from Dr. Charles B. Huggins of the Department of Surgery. A solution of the following composition was incubated for various periods at

TABLE I

Hydrolysis of l-Phosphomalic Acid in 1 N H₂SO₄ at 100°

Each aliquot contains 29.8 γ of esterified P.

Time	Inorganic phosphorus released	Hydrolysis
<i>min.</i>	γ	<i>per cent</i>
10	0.2	0.7
60	2.3	7.7
120	4.4	15
180	5.6	19

TABLE II

Action of Acid Phosphatase on l-Phosphomalic Acid at Room Temperature (25°)

Each aliquot contains 23.0 γ of esterified P.

Time	Inorganic phosphorus released	Hydrolysis
<i>min.</i>	γ	<i>per cent</i>
30	7.0	30
60	11.5	50
180	15.0	65

room temperature: 1.0 ml. of *l*-phosphomalic acid (3.7 micromoles or 115 γ of esterified P and 10 γ of inorganic P), 1.0 ml. of diluted acid phosphatase preparation from human semen (containing 105 γ of inorganic P), 5.0 ml. of acetate buffer, pH 5.0.

The reaction was stopped at different time intervals by addition of 2.5 ml. of 20 per cent trichloroacetic acid and inorganic phosphate determined on aliquots of the filtrate. Table II shows the extent of dephosphorylation at various time intervals.

SUMMARY

Phosphomalic acid was synthesized by phosphorylating diethyl malate with diphenylphosphoryl chloride, followed by removal of the phenyl

groups by catalytic hydrogenolysis and saponification of the resulting ethyl ester. The *d*, *l*, and *dl* enantiomorphs of phosphomalic acid were prepared as barium salts. Crystalline brucine salts of phosphomalic acid were also obtained.

Phosphomalic acid was found to be relatively stable to acid and alkali, but easily hydrolyzed by the action of acid phosphatase.

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THE USE OF RANEY'S NICKEL IN A DIFFERENTIAL ASSAY FOR OXYBIOTIN AND BIOTIN*

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A method for the determination of oxybiotin in the presence of biotin in biological materials has recently been described (1). This differential assay was based upon the stability of oxybiotin towards dilute potassium permanganate and the inactivation of biotin by this reagent. This paper describes another differential assay which is based on the contrasting behavior of biotin and oxybiotin towards Raney's nickel catalyst.

Du Vigneaud *et al.* (2), applying the Mozingo reaction (3), have shown that biotin undergoes hydrogenolysis when treated with Raney's nickel, and is thereby converted into desthiobiotin. It could be expected that the tetrahydrofuran moiety of oxybiotin would be resistant to this treatment. This was actually found to be the case.

In materials containing both biotin and oxybiotin, treatment with Raney's nickel converts the biotin into desthiobiotin without affecting the oxybiotin.¹ Desthiobiotin is inactive for *Lactobacillus arabinosus* (5). Therefore, the activity remaining after treatment with Raney's nickel is due to the oxybiotin present. These considerations served as the basis for a quantitative method for the determination of oxybiotin in the presence of biotin. A similar procedure has already been applied by Stokes and Gunness (6) to demonstrate the conversion of desthiobiotin to biotin.

Methods and Materials

Preparation of Solutions for Assay—Solutions of *d*-biotin and *dl*-oxybiotin were prepared in 0.1 *N* sodium hydroxide at concentrations of 2 and 4 millimicrograms per ml., respectively. A solution containing 1 millimicrogram of biotin and 2 millimicrograms of oxybiotin per ml. was also employed.

The natural materials were first hydrolyzed with acid, according to standard procedures. They were then neutralized and additional sodium hydroxide added to a concentration of approximately 0.1 *N*. The final

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¹ *dl*-Oxybiotin has 50 per cent the activity of *d*-biotin for *Lactobacillus arabinosus* (4).

concentration of biotin in the alkaline extracts should not exceed 2 millimicrograms per ml.

Preparation of Catalyst—A 200 mg. sample of Raney's nickel alloy was suspended in 5.7 ml. of 4 per cent sodium hydroxide in a 50 ml. volumetric flask and heated for 45 minutes on a steam bath. The liquid was decanted, 5.7 ml. of 4 per cent sodium hydroxide were added, and the suspension again heated for 15 minutes. The catalyst was washed by decantation with three 5 ml. portions of water and was then ready for use.

Inactivation Procedure—A solution prepared as described above (10 ml.) was added to the catalyst and the suspension was mechanically shaken in

TABLE I
*Differential Behavior of Biotin and Oxybiotin towards Raney's Nickel in Pure Solutions and Yeast Extracts**

Material	After Raney's nickel treatment (4.0 millimicrograms before treatment)
	millimicrograms per ml.
<i>d</i> -Biotin	0.0
<i>dl</i> -Oxybiotin	4.0
<i>d</i> -Biotin + <i>dl</i> -oxybiotin†	2.0
Yeast extract	0.0
" " + <i>d</i> -biotin‡	0.0
" " + <i>dl</i> -oxybiotin§	2.0

* All activities are expressed as oxybiotin equivalents. These equivalencies are obtained by referring growth activities to an oxybiotin standard curve.

† A solution containing 1 millimicrogram of *d* biotin and 2 millimicrograms of *dl*-oxybiotin per ml.

‡ The yeast extract was diluted with an equal volume of a solution containing 2 millimicrograms of *d*-biotin per ml.

§ The yeast extract was diluted with an equal volume of a solution containing 4 millimicrograms of *dl*-oxybiotin per ml.

a boiling water bath for 10 minutes, made up to volume with distilled water, and filtered. 10 mg. of Raney's nickel alloy should be employed for each millimicrogram of *d*-biotin present. It is essential to conduct the inactivation procedure in an alkaline solution, since at neutrality oxybiotin is strongly adsorbed by Raney's nickel catalyst. 25 ml. of the filtrate were placed in a 50 ml. volumetric flask, adjusted to pH 6.8 with 0.5 N hydrochloric acid with brom-thymol blue as an internal indicator, and made up to volume with distilled water. The growth-promoting activity of this solution was then determined with *Lactobacillus arabinosus* (7). The original activity of the solution was determined by applying the identical procedure in the absence of the catalyst. The results obtained are given in Table I.

SUMMARY

The results reported in this paper may be summarized as follows.

1. Biotin in pure solution or in the presence of yeast extract is quantitatively inactivated by Raney's nickel.

2. Under similar conditions, oxybiotin is unaffected by this treatment.

3. The activity remaining after treatment of a mixture of biotin and oxybiotin is due to the oxybiotin present.

4. All of the activity present in a yeast extract is inactivated by the treatment with Raney's nickel.

A quantitative procedure for the determination of oxybiotin in the presence of biotin in natural materials has been developed.

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THE METABOLISM OF OXYBIOTIN IN YEAST*

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The demonstration of the marked biological activity of oxybiotin (1-4), the oxygen analogue of biotin, has focused attention upon the mode of action of this compound. It became of particular interest to determine whether oxybiotin possesses intrinsic activity or whether its biological potency is due to its conversion into biotin. The evidence in the literature is conflicting on this point. Hofmann and Winnick (5), employing a differential assay procedure based upon the selective destruction of biotin activity by dilute potassium permanganate, have demonstrated that *Saccharomyces cerevisiae* and *Rhizobium trifolii* utilize oxybiotin as such and do not convert it into biotin. On the other hand, Rubin *et al.* (6) have presented data from balance studies on *S. cerevisiae*, which indicates that O-heterobiotin¹ was converted into biotin or some other compound which possessed more activity for *S. cerevisiae* than did oxybiotin.

The experiments described in this paper were designed to clarify this controversial point. The yeast balance studies of Rubin *et al.* (6) were repeated under conditions identical with those employed by these authors. In addition, three differential assay procedures for the determination of oxybiotin in the presence of biotin have been utilized to ascertain whether cells of *Saccharomyces cerevisiae* grown in the presence of oxybiotin contain biotin. These four independent experimental approaches led to the same conclusion; namely, that *Saccharomyces cerevisiae* does not convert oxybiotin into biotin.

Balance Studies

These experiments were conducted in an effort to repeat the observations of Rubin *et al.* (6). *Saccharomyces cerevisiae* was grown in the presence of *dl*-oxybiotin and the *dl*-oxybiotin content of the harvested cells and medium was determined by *S. cerevisiae* assay (8). Since for yeast *dl*-oxybiotin is only 25 per cent as active as *d*-biotin, any conversion of oxybiotin into biotin during growth would lead to an increase in total oxybiotin equivalents² and an apparent recovery of added oxybiotin ex-

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¹ O-Heterobiotin has been shown to be identical with oxybiotin (7).

² Since in the present studies we are dealing with mixtures of biotin and oxybiotin,

ceeding 100 per cent. Such high recoveries were reported by Rubin *et al.* and served as a basis for their conclusion that oxybiotin was converted into biotin or a vitamer of similar activity.

Saccharomyces cerevisiae was grown in 250 ml. Erlenmeyer flasks containing 40 ml. of the Snell medium (9). Varying amounts, *i.e.* 10, 25, 50, or 100 millimicrograms, of *dl*-oxybiotin were added to each flask. In other experiments, 2 and 8 millimicrograms of *d*-biotin were employed. Distilled water was added to a final volume of 47 ml. After autoclaving for 10 minutes, the contents were cooled and seeded with 1 ml. of a suspension of *Saccharomyces cerevisiae* 139³ prepared by suspending cells from a 24 hour culture grown on Difco wort agar in sufficient sterile distilled water to give a concentration of 0.55 mg. of dry yeast per ml.⁴ This amount of dry yeast contains 0.60 millimicrogram of *d*-biotin, which is equivalent to 2.4 millimicrograms of *dl*-oxybiotin in yeast growth-promoting activity. Since this amount of oxybiotin activity represents a considerable proportion of the total oxybiotin added in the 10 millimicrogram balance studies, experiments with this level of oxybiotin were also performed in which the inoculum consisted of 0.01 mg. of dry yeast. The biotin content of this quantity of yeast is negligible. All experiments were conducted in duplicate.

After incubation at 30° for 18 hours, the cells were harvested by centrifugation. The supernatant medium was autoclaved for 15 minutes and assayed without further treatment. The cells were autoclaved for 1 hour with hydrochloric acid. In order to determine the optimum extraction procedure, the effects of varying amounts and concentrations of acid were studied. For the 10 millimicrogram studies, 10 ml. of 2 N hydrochloric acid yielded maximum extraction values. In the remainder of the balance studies, the maximum yield of growth factor was obtained when 5 ml. of 6 N hydrochloric acid were employed. These conditions were utilized to obtain the results reported in the present paper. In our early experiments, the cells were hydrolyzed with sulfuric acid. The same results in the balance studies were obtained with both acids.

The acid hydrolysates were adjusted to pH 4.0 with sodium hydroxide, diluted to a concentration of approximately 0.2 millimicrogram of oxybiotin equivalents per ml., and filtered. Both the clear filtrates and the

it has been found desirable to express all activities in terms of oxybiotin equivalents. These equivalents are obtained by referring growth activity to an oxybiotin standard curve.

³ Obtained from the American Type Culture Collection.

⁴ The yeast concentration was determined in an Evelyn photocolormeter from a calibration curve relating turbidity reading to dry weight of yeast. The dry matter content of these cells was 23 per cent by weight.

autoclaved medium were then assayed for their oxybiotin activity by the *Saccharomyces cerevisiae* method of Hertz (8).^{5,6} In those balance studies in which biotin was added, the biotin content was calculated from a biotin standard curve. Satisfactory recoveries of biotin and oxybiotin were obtained when these compounds were added to the harvested yeast cells before acid hydrolysis.

The results of the oxybiotin balance studies are given in Table I. The percentage recovery of added oxybiotin was less than 100 and demonstrates that oxybiotin is not converted into biotin or any other compound with more activity for *Saccharomyces cerevisiae*. In two balance experi-

TABLE I
Balance Studies with *Saccharomyces cerevisiae*

Oxybiotin added to medium	No. of experiments	Type of inoculum	Oxybiotin equivalents found in		Recovery of added oxybiotin
			Cells*	Medium	
millimicrograms			millimicrograms	millimicrogram	per cent
10	7	Low†	8.7 (6.0-10.0)	1	97
10	13	High‡	8.4 (7.3-9.6)	1	94
25	4	"	21.4 (18.5-23.3)	1	90
50	4	"	43.3 (40-46)	1	89
100	4	"	83.3 (80-86)	1	84

* Average values are given with ranges in parentheses. In the *high* inoculum experiments these are corrected values and are the oxybiotin equivalents observed minus the oxybiotin equivalents (2.4 millimicrograms) furnished by the biotin present in the inoculum.

† Flasks seeded with 0.01 mg. of dry yeast.

‡ Flasks seeded with 0.55 mg. of dry yeast.

ments with *d*-biotin, recoveries of 105 and 85 per cent of the added biotin were observed.

Differential Assay Procedures

It appeared desirable to confirm the results of the balance studies by demonstrating the absence of biotin in cells of *Saccharomyces cerevisiae*

⁵ The assays were conducted in 50 ml. Erlenmeyer flasks at 30°.

⁶ It should be pointed out that the hydrolysates contain both *d*-biotin (from the inoculum) and *dl*-oxybiotin. Since with *Saccharomyces cerevisiae* the growth curves of these two compounds are different in shape (2), different activity ratios at various portions of the curves are observed. However, the shapes of the growth curves are identical up to 1 millimicrogram of oxybiotin equivalents per flask. Suitable assays can, therefore, be obtained if the amount of oxybiotin equivalents added per assay flask is kept below 1 millimicrogram.

grown on oxybiotin. Methods permitting the differentiation of biotin and oxybiotin were necessary for this purpose. Three such differential assay procedures have been devised in our laboratories and were applied to the acid hydrolysates of cells of *Saccharomyces cerevisiae* grown in the presence of varying amounts of oxybiotin. The methods employed in growing the cells and obtaining the acid hydrolysates have been described in the previous section. Each flask was seeded with 0.55 mg. of dry yeast. It was necessary to combine the harvested cells from several flasks and hydrolyze them with proportionally increased amounts of 2 N or 6 N hydrochloric acid in order to obtain enough material for analysis.

Permanganate Method (5)—This method is based upon the fact that biotin is converted by dilute potassium permanganate into biotin sulfone, which is inactive for *Saccharomyces cerevisiae* in the amounts present under our assay conditions. In contrast, this treatment causes only a slight destruction of oxybiotin. Therefore, any growth activity for *Saccharomyces cerevisiae* remaining after treatment of a mixture of biotin and oxybiotin with permanganate is due to the oxybiotin present.

The hydrochloric acid hydrolysates prepared from the harvested yeast cells were adjusted to pH 1.0 with sodium hydroxide, diluted to a concentration of approximately 2 millimicrograms of oxybiotin equivalents per ml., and filtered. 3 ml. of the filtrate were treated with 6 ml. of 0.01 N potassium permanganate and allowed to stand at room temperature for 5 minutes. After decolorization of the excess permanganate with 0.05 M sodium bisulfite, the solution was adjusted to pH 4.0 with sodium hydroxide, and diluted to 25 ml. with distilled water. The growth activity for *Saccharomyces cerevisiae* was then determined (8). The same procedure was applied to 3 ml. of a 0.1 N hydrochloric acid solution containing 10 millimicrograms of *dl*-oxybiotin. The growth curve obtained with this permanganate-treated oxybiotin solution was used as the reference standard. This treatment of oxybiotin with permanganate resulted in a 10 per cent loss in activity.⁷ When a solution of oxybiotin was added to the

⁷ More extensive experience with this differential assay (5) has demonstrated that the acid employed markedly influences the stability of oxybiotin towards potassium permanganate. Whereas oxybiotin is only slightly inactivated in 0.1 N hydrochloric acid solution, it is completely inactivated when 0.1 N sulfuric acid is employed. No explanation can be offered at the present time for this behavior of the oxybiotin molecule. It is interesting to note, however, that the stability of oxybiotin in the presence of a yeast hydrolysate is independent of the acid employed in the preparation of the hydrolysate. The same results were obtained when either hydrochloric acid or sulfuric acid was used to hydrolyze the yeast cells and the hydrolysate treated with potassium permanganate. A 90 per cent recovery of the oxybiotin added to a sulfuric acid yeast hydrolysate was obtained following permanganate treatment.

original yeast filtrate and the mixture treated with permanganate, as described above, a 90 per cent recovery of the added oxybiotin was obtained. Added biotin was completely destroyed under these conditions.

In order to determine the oxybiotin equivalency previous to permanganate inactivation, 3 ml. of the yeast filtrate were treated as shown, with the exception that the permanganate and bisulfite additions were omitted.

TABLE II

Effect of Permanganate and Raney's Nickel Catalyst upon Growth Activity of Hydrolysates Prepared from Saccharomyces cerevisiae Grown in Presence of Oxybiotin

The values are given in millimicrograms.

Oxybiotin added to medium	Oxybiotin equivalents in cell,		
	Before treatment*	After permanganate treatment	After Raney's nickel treatment
10	8.0	7.6	
	9.1	9.0	
	6.8	7.0	
	7.3	7.2	
	9.5		9.5
	7.5		7.4
50	42	38	
	46	43	
	40		37
	45		45
100	85	82	
	80	83	
	86		86
	82		75

* Oxybiotin equivalents observed minus the oxybiotin equivalents furnished by the biotin present in the inoculum. In separate experiments, it was demonstrated that the biotin in the inoculum was completely inactivated by both permanganate and Raney's nickel catalyst.

In this case the oxybiotin equivalency was determined by reference to a non-potassium permanganate-treated oxybiotin standard.

The results obtained are shown in Table II. It can be seen that the growth activity present in cells of *Saccharomyces cerevisiae* grown on oxybiotin is not significantly affected by permanganate treatment. The absence of biotin in these cells is, therefore, demonstrated.

Raney's Nickel Method—This differential assay procedure is described in the preceding paper (10) and is dependent upon the fact that treatment with Raney's nickel, while without effect upon oxybiotin, converts biotin

into desthiobiotin, which is inactive for *Lactobacillus arabinosus*. *dl*-Oxybiotin is 50 per cent as active as *d*-biotin for *Lactobacillus arabinosus*.

The growth activity of the acid hydrolysates of *Saccharomyces cerevisiae* was determined by *Lactobacillus arabinosus* assay both before and after treatment with Raney's nickel and was expressed as oxybiotin equivalents. Since biotin is inactivated by Raney's nickel, any activity remaining after such treatment must, perforce, be due to the oxybiotin present. Biotin added to the acid hydrolysates was completely inactivated by treatment with Raney's nickel, while added oxybiotin was unaffected.

The results of these studies are likewise given in Table II. Since the growth activity of the extracts from cells of *Saccharomyces cerevisiae* grown in the presence of oxybiotin was not affected by Raney's nickel catalyst, it can be concluded that these extracts do not contain any biotin. On the other hand, the growth activity of extracts from cells grown in the presence of biotin was completely inactivated by Raney's nickel catalyst.

Differential Growth Inhibition by γ -(3,4-Ureylenecyclohexyl)-butyric Acid—We have previously reported (11) that the activity of *dl*-oxybiotin for *Lactobacillus arabinosus* can be completely inhibited by amounts of γ -(3,4-ureylenecyclohexyl)-butyric acid which have only a slight effect upon the activity of equivalent amounts of *d*-biotin. This selective inhibition has been utilized as a basis for the differential assay of biotin and oxybiotin. In the presence of γ -(3,4-ureylenecyclohexyl)-butyric acid the growth-promoting effect of mixtures of biotin and oxybiotin is due only to the biotin present. The decrease in activity observed when such mixtures are assayed in the presence of the inhibitor is therefore due to the oxybiotin content. Complete growth inhibition is indicative of the absence of biotin in a suspected mixture.

Acid hydrolysates of *Saccharomyces cerevisiae* cells grown in the presence of 10, 50, or 100 millimicrograms of oxybiotin were adjusted to pH 6.8 with sodium hydroxide, filtered, and diluted to a concentration of approximately 0.4 millimicrogram of oxybiotin equivalents per ml. (determined by *Lactobacillus arabinosus* assay with an oxybiotin standard curve). The activities of aliquots of these extracts containing from 0.5 to 2.0 millimicrograms of oxybiotin equivalents were then determined in the presence of 150 γ of γ -(3,4-ureylenecyclohexyl)-butyric acid. Complete inhibition of growth at all levels was noted in the extracts prepared from cells grown on 50 or 100 millimicrograms of oxybiotin. In these aliquots the oxybiotin equivalents furnished by the biotin in the inoculum were below detectable limits. The slight growth activity remaining in the extracts from the 10 millimicrogram studies could be attributed to the biotin furnished by the inoculum which represents 10 per cent of the total growth-promoting activity of the extracts for *Lactobacillus arabinosus*.

The inability of these extracts to support the growth of *Lactobacillus arabinosus* in the presence of γ -(3,4-ureylenecyclohexyl)-butyric acid again demonstrates the absence of biotin.

DISCUSSION

The balance experiments described in this paper demonstrate clearly that oxybiotin is not converted into biotin during the growth of *Saccharomyces cerevisiae* 139. These results are in direct contrast to those obtained by Rubin *et al.* in identical experiments. The data presented by these authors indicate complete conversion of oxybiotin into biotin at a low level of added oxybiotin. We can offer no explanation for this extreme variance in results. The failure of Rubin *et al.* to take into account the biotin furnished by the inoculum would lead to an apparent recovery of approximately 120 per cent in the 10 millimicrogram balance studies. This figure, however, falls far short of the 200 per cent recovery observed by these workers.

The analyses of yeast hydrolysates by three distinct differential assay methods have further demonstrated the absence of biotin in cells of *Saccharomyces cerevisiae* grown on oxybiotin. These results are in accord with those obtained in our balance experiments and confirm the work of Hofmann and Winnick (5). These data provide unequivocal proof for the absence of a measurable equilibrium state between biotin and oxybiotin in *Saccharomyces cerevisiae* 139. The biological activity of oxybiotin for this yeast is, therefore, an intrinsic property of the molecule.

SUMMARY AND CONCLUSIONS

1. In balance experiments with *Saccharomyces cerevisiae* 139 grown in the presence of varying amounts of *dl*-oxybiotin approximately 100 per cent of the added oxybiotin was recovered.

2. Three independent differential assay methods have been utilized to demonstrate the absence of biotin in cells of *Saccharomyces cerevisiae* grown on oxybiotin.

3. *Saccharomyces cerevisiae* 139 does not convert oxybiotin into biotin and the biological activity of oxybiotin, the oxygen analogue of biotin, is an inherent property of the molecule.

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THE ACTION OF PROTEOLYTIC ENZYMES AND PROTAMINASE ON SALMINE SULFATE

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The possible rôle of protamines as intermediates in the enzymatic hydrolysis of proteins has held the interest of a number of investigators since the discovery of these basic polypeptides by Miescher (1, 2) in the sperm of fish. This class of polypeptides resembles genuine proteins quite closely with respect to molecular weight (approximately 3000) and presence of peptide linkages connecting the various amino acid residues, yet differs from proteins in that only a few species of amino acids are present. Therefore protamines were thought to lend themselves admirably to the study of substrate specificity of proteolytic enzymes. Moreover, it was felt that the structure of protamines could be elucidated further by the use of proteolytic enzymes. It was with this in mind that Kossel and Matthews (3) reported that salmine and sturin were hydrolyzed by crude trypsin preparations but not by pepsin, and Waldschmidt-Leitz and Harteneck (4) reported that erepsin did not utilize protamines as substrates. These results indicate that protaminolytic activity is associated with the trypsin fraction of pancreatic extracts. Waldschmidt-Leitz and coworkers (5, 6) reported the isolation of an enzyme which was capable of hydrolyzing protamines, though unable to act on proteins. Their findings led them to abandon their former hypothesis that trypsin could split protamines. Northrop (7), however, was unable to separate protaminase from chymotrypsin.

The isolation of crystalline trypsin and chymotrypsin by Northrop and Kunitz (8, 9) enabled Waldschmidt-Leitz and Akabori (10) to reinvestigate the action of proteinases on protamines and their degradation products. Thus, Waldschmidt-Leitz and Akabori compared their own proteinase preparation with the crystalline enzyme preparation of Northrop and Kunitz (8, 11) and studied the effect of these enzymes with respect to the liberation of amino nitrogen on casein, sturin, clupein, and clupean. Their findings indicate that the non-crystalline proteinase contained impurities and that clupean,¹ the degradation product of clupein, is split more extensively than the native protamine. In 1935 Holter, Kunitz, and

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¹ Waldschmidt-Leitz and Kofranyi have characterized clupean as a split-product of clupein. It is thought to contain three tripeptides and two dipeptides (12).

Northrop (13) repeated the same experiments of Waldschmidt-Leitz and Akabori, limiting themselves to clupean and employing crystalline trypsin and chymotrypsin prepared by an improved method (14) which successfully removed impurities present in earlier crystalline preparations. These experiments largely confirmed Waldschmidt-Leitz and Akabori's data on clupean.

Furthermore, Weil (15), using a somewhat different procedure for the preparation of protaminase, presented evidence that proteinase action on protamines ceased at the clupean stage, but that protaminase was capable of hydrolyzing protamines as well as their decomposition products, such as clupean.

Since the work of Holter, Kunitz, and Northrop deals only with the effect of improved crystalline trypsin and chymotrypsin on a protamine degradation product, *i. e.* clupean, it was thought worth while to present

TABLE I
Calculation of Purity of Authors' Preparation by Comparison with Block and Bolling's Data

	Block and Bolling (16)	Authors' data	Purity of authors' preparation
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Arginine	68.57	53.5*	78.0
Kjeldahl N .	24.46	20.00	81.60

* Arginine determined after complete hydrolysis of salmine according to the method described in this paper, except that protaminase was in excess.

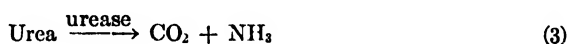
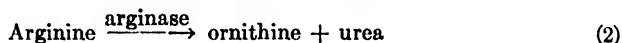
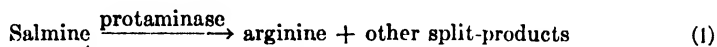
in this paper data concerning the effect of improved crystalline enzyme preparations on a *native* protamine, namely salmine. It should be pointed out that the results obtained with salmine are not directly comparable to those obtained with clupein, since these two protamines differ slightly as regards their digestion by protaminase. Comparable data for the digestion of these protamines by proteinases are not available.

Determination of Protaminolytic Activity

Salmine sulfate (Eli Lilly and Company) was used as substrate for the protaminolytic enzyme. The purity (80 per cent) of this salmine preparation was calculated from its arginine and nitrogen content with Block and Bolling's (16) data for comparison (Table I).

Salmine sulfate can be hydrolyzed to its component amino acids by refluxing 24 hours with dilute sulfuric acid. This can also be accomplished enzymatically under certain conditions. The method which was formerly used for the determination of the extent of protamine hydrolysis consisted

in the measurement of the increase in free amino nitrogen. Because a more specific method for determination of protaminase activity was desired and because arginine constitutes 64.57 per cent of salmine sulfate, the amount of free arginine liberated by protaminase was used as a criterion for protaminolytic activity. Three enzyme-catalyzed reactions are involved in the analytical procedure which ultimately yields measurable carbon dioxide.



The specificity of this method might be influenced by the presence of substrates other than arginine to yield urea and ornithine. However, such interference seems unlikely in view of the findings of Waldschmidt-Leitz and Kofranyi (12) that arginase did not affect any of the protamine digestion products, regardless of size, except free arginine. Furthermore, it has been shown that a number of derivatives of arginine, as well as several arginine-like compounds, are not hydrolyzed by arginase. The following examples may be cited: DL- ϵ -guanido- α -aminocaproic acid (17), γ -guanidobutyric acid (18-20) δ -guanidovaleric acid (21), argininic acid (21), α ,N-methylarginine (22), α ,N-benzoylarginine (18, 23), and argininamide (23). Contrary to Waldschmidt-Leitz and Kofranyi (12), Edlbacher and Burchard (24) have reported the splitting of a degradation product of clupein, isolated by Kossel and Staudt (25) and identified as arginylarginine, by an arginase preparation free of dipeptidase activity. The preparation of Kossel and Staudt probably contained impurities which were eliminated in methods developed later for the isolation of arginylarginine, in which either acid hydrolysis (24-26) or tryptic digestion (27) of clupean was used. The work of Edlbacher and Burchard (24) was confirmed recently by Felix and Schuberth (28) who used well defined and carefully analyzed preparations of arginylarginine. Since in the experiments to be reported no urea formation could be observed when trypsin, chymotrypsin, or pepsin was added to salmine, this protamine appears to behave in a manner similar to that described by Waldschmidt-Leitz and Kofranyi (12) for clupein, whose proteolytic digestion products are not hydrolyzed by arginase.

The formation of arginylarginine in the course of the hydrolysis of protamine by protaminase could conceivably produce misleading results in the method here reported. But the formation of arginylarginine under these conditions appears quite unlikely, since, according to Waldschmidt-Leitz

and Kofranyi (12), protaminase produces only free arginine from clupein, as is evidenced by the isolation of arginine flavianate.

The determination of protaminolytic activity is carried out as follows: 50 mg. of salmine sulfate (80 per cent pure) were dissolved in 4 ml. of 0.3 M phosphate buffer, pH 7.7, and the enzyme solution containing protaminase was added in suitable concentration. The final volume was made up to 10 ml. with water and the solution incubated at 37° for 24 hours. At the end of the incubation period the solution was heated to 100° in a water bath for 15 minutes and the coagulated proteins centrifuged off and discarded. 5 ml. of the supernatant were added to 1 ml. of arginase solution (prepared according to Richards and Hellerman (29)), after addition of 4 ml. of 0.3 M phosphate buffer, pH 7.7. This solution was incubated 5 hours at 37° and then was heated to 100° in a water bath for 15 minutes. After centrifugation, the urea formed due to arginase action was estimated manometrically with urease, 10 mg. in each vessel (Squibb commercial preparation), in Warburg manometers and vessels. Theoretical amounts of arginine could be recovered when arginine was hydrolyzed with arginase and urea determined manometrically. Previous investigators have described the manometric determination of urea, *e.g.* Krebs and Henseleit (30), and a manometric assay of arginase activity has been reported (31).

It is essential for the success of the method outlined in the preceding paragraphs that arginase and urease are present in excess, so that reaction (1) is the limiting factor in this series of reactions. Furthermore it was necessary to ascertain that arginase and urease were devoid of any protaminolytic activity. This was found to be true here.

Effect of Proteolytic Enzymes on Salmine Sulfate

Three crystalline proteolytic enzymes were investigated, namely trypsin (32), chymotrypsin (32), and pepsin (33). Tryptic and chymotryptic digestions were carried out at pH 7.7, whereas peptic digestion was carried out at pH 2.0. The proteolytic activities were determined by the method of Anson (34). Amino nitrogen was determined according to the gasometric Van Slyke method.

It was found that trypsin and chymotrypsin were capable of hydrolyzing salmine and that in the process of hydrolysis one-half of the total amino nitrogen was liberated (Table II). These values are slightly lower (about 10 per cent) than those obtained by Waldschmidt-Leitz and Akabori (10) for the digestion of clupein by trypsin (with the older method of preparation (8)). On the other hand, the amount of amino nitrogen liberated by chymotrypsin is approximately 10 per cent higher than the values obtained by Waldschmidt-Leitz and Akabori (10) with less pure chymotrypsin preparations (11) and clupein as substrate. Pepsin, however, did not

affect salmine sulfate. None of the above proteolytic reaction products was susceptible to arginase action, indicating the absence of free arginine, or possibly arginylarginine. These results seem to be in disagreement with those of Felix *et al.* (27) who have reported the isolation of arginyl-arginine from tryptic digests of clupein methyl ester hydrochloride. This

TABLE II
*Liberation of Amino Nitrogen upon Digestion of Salmine Sulfate by
Proteolytic Enzymes*

Enzyme	Amino nitrogen per 10 mg salmine
	mg
Chymotrypsin	0.204
Trypsin	0.252
Pepsin	0.000

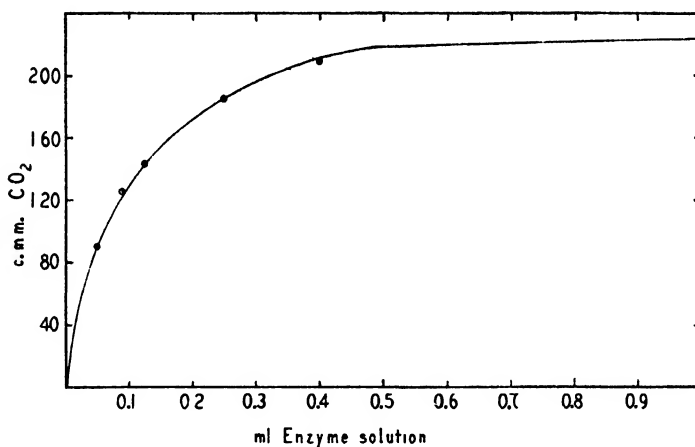


FIG. 1. Determination of protaminase. The CO₂ values represent the total amount liberated upon digestion of 50 mg. of salmine sulfate according to the procedure described in the text. Protaminase solution, 0.38 mg. of protein per ml

discrepancy may be due to the contamination of their rather crude trypsin preparation by protaminase.

A commercial trypsin preparation (Wilson and Company) was used as a source of protaminase. This powder was extracted with 0.25 N H₂SO₄ and yielded protaminase activity which may be measured as shown in Fig. 1.

The data presented in Fig. 1 indicated that proteolytic enzymes were capable of using salmine sulfate as a substrate, but that they were unable to hydrolyze salmine completely, since only one-half of the amino nitrogen (calculated from the arginine content) was liberated.

It appears possible that the action of proteolytic enzymes on salmine precedes that of protaminase; so that protaminase would act only upon the proteolytic degradation product of salmine. Such a possibility seems indicated by the fact that proteolytic enzymes and protaminase are closely associated and difficult to separate.

Separation of Proteolytic and Protaminolytic Activity

An attempt was made to separate protaminase from the proteinases and peptidases by a variety of procedures. (1) Fractionations with $(\text{NH}_4)_2\text{SO}_4$ or alcohol were unsuccessful. (2) Removal of peptidases with alumina C gel at pH 3.8 and 7.0 was performed. Then adsorption with alumina A of protaminase was attempted but was unsuccessful. Weil (15) stated that he was able to separate protaminolytic and proteolytic activity by this means. (3) Lastly adsorption of proteinases on egg albumin and subsequent precipitation with acetone did not separate protaminase from the proteinases as reported by Waldschmidt-Leitz and Kofranyi (6).

The criteria for separation employed here differ somewhat from those previously used. Protaminolytic activity was determined by the method described above, whereas proteolytic activity was determined by the method of Anson (34). The difference between the results obtained may be due to the higher specificity of the methods used here or, perhaps, to the difference in substrate employed in the measurements of proteolytic activity.

SUMMARY

1. A method for the measurement of protaminase activity is presented.
2. Crystalline trypsin and crystalline chymotrypsin hydrolyzed salmine sulfate and liberated one-half of the arginine amino nitrogen. Crystalline pepsin caused no hydrolysis.
3. None of the proteolytic enzymes was capable of complete hydrolysis of salmine sulfate to free arginine.
4. Protaminase was found to yield free arginine from salmine.
5. The failure to separate protaminolytic from proteolytic activity has been reported.

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THE ACONITE ALKALOIDS

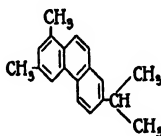
XVI. ON STAPHISINE AND THE HYDROCARBON OBTAINED FROM ITS DEHYDROGENATION

BY CHARLES F. HUEBNER AND WALTER A. JACOBS

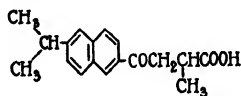
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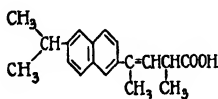
Staphisine, atisine, and napelline, members of the less toxic group of the aconite alkaloids, have been shown to yield phenanthrene hydrocarbons on selenium dehydrogenation (1-4). The hydrocarbon ($C_{19}H_{20}$) obtained along with pimanthrene and other hydrocarbons from staphisine, must represent a large fragment of the original molecule based either on the monomolecular or dimolecular formulas of the alkaloid previously discussed (1). Its identification has therefore been of special interest. Oxidative studies (4) have already suggested that the substance could be a dimethylisopropylphenanthrene, the possible structures of which were limited to three.



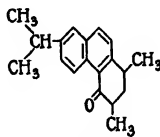
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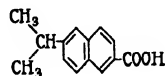
II



III



IV



V

Synthetic studies have now furnished convincing evidence that it is one of these; namely, 1,3-dimethyl-7-isopropylphenanthrene (3-methylretene) (Formula I). The hydrocarbon obtained from the alkaloid as well as its picate and trinitrobenzene derivative have appeared indistinguishable from the synthetic substances.

The synthesis of 1,3-dimethyl-7-isopropylphenanthrene has followed closely the plan used by Haworth and Bolam for the preparation of 1,3,7-trimethylphenanthrene (5). The initial step for this purpose was the substitution of 2-isopropylphenanthrene for 2-methylphenanthrene for con-

densation with methylsuccinic anhydride and aluminum chloride to yield *2-(6-isopropyl-2-naphthoyl)isobutyric acid* (Formula II). The *methyl ester* of this acid with methylmagnesium iodide and the accompanying loss of H_2O and saponification gave *1,3-dimethyl-3-(6-isopropyl-2-naphthyl) Δ^2 -butenoic acid* (Formula III). Since the succeeding steps which finally resulted in the desired phenanthrene derivative exactly paralleled the synthesis described by Haworth and Bolam, and because of limited material, no attempt was made to purify the intermediate substances for characterization. These steps and the procedures used were as follows.

Reduction of the above butenoic acid with hydriodic acid and phosphorus gave *1,3-dimethyl-3-(6-isopropyl-2-naphthyl)butyric acid*. The crude acid isolated was directly cyclized with H_2SO_4 to the ketone *4-oxo-1,3-dimethyl-7-isopropyl-1,2,3,4-tetrahydrophenanthrene* (Formula IV). The crude ketone on Clemmensen reduction yielded *1,3-dimethyl-7-isopropyl-1,2,3,4-tetrahydrophenanthrene*. The latter, also without purification, was dehydrogenated with selenium to *1,3-dimethyl-7-isopropylphenanthrene*. For further characterization of the hydrocarbon and comparison with the substances obtained from staphisine, its *picrate* and *trinitrobenzene derivative* were prepared.

As a check on the position occupied by the methylsuccinoyl group in the above *2-(6-isopropyl-2-naphthoyl)isobutyric acid*, it was shown that the latter on oxidation with potassium ferrieyanide yielded an acid which approached in properties the *6-isopropyl-2-naphthoic acid* (Formula V) described by Haworth (6). This was confirmed by condensation of *2-isopropyl-naphthalene* with propionyl chloride to form *ethyl-(6-isopropyl-2-naphthyl) ketone*, since the latter on subsequent oxidation with hypochlorite also yielded the same *6-isopropyl-2-naphthoic acid*.

The identification of the $C_{19}H_{20}$ hydrocarbon from staphisine as *1,3-dimethyl-7-isopropylphenanthrene* gives additional support, at least as far as the 1-methyl and 7-isopropyl groups are concerned, to the suggested general diterpenoid nature of this alkaloid, and therefore of other aconite alkaloids. As we shall report elsewhere, evidence has been obtained that pimanthrene, as in the case of staphisine, is a product of the dehydrogenation of hetisine. The origin of the 3-methyl group in the $C_{19}H_{20}$ hydrocarbon from staphisine, however, requires an explanation and will be a subject for further study. A diterpenoid structure could account for 20 carbon atoms of the alkaloid. The group which appears in the N-alkyl determination has been definitely identified as methyl.¹ If the simple monomolecular alkaloid possesses only these carbon atoms, it would be a

¹ The so called N-alkyl groups present in the *Aconitum* alkaloids which we have been able to study as in the case of aconitine are N-ethyl groups. This will be described elsewhere.

C_{21} derivative and the dimolecular alkaloid would require a C_{42} formula. As a matter of fact, the analytical data already presented for staphisine (1) are essentially as satisfactory for a $C_{42}H_{60}ON_2$ formula derived by loss of H_2O between two $C_{21}H_{31}ON$ alkaloid molecules (Table I). A $C_{42}H_{58}ON_2$ formula is also a possibility, in which case a carbon to carbon bond between non-identical moieties could be present. If this bond is shared by the 3-phenanthrene carbon atom of one of the moieties, the 3-methyl group above might have its origin in a carbon atom of the second half. As another possibility, if the N atom of the monomolecular alkaloid is attached in the environment of carbon atom 3, a rearrangement of its N-methyl group to

TABLE I
Staphisine and Derivatives

Substance	Calculated on basis of						Found	
	$C_{21}H_{31}ON_2$		$C_{42}H_{60}ON_2$		$C_{42}H_{58}ON_2$		C	H
	C	H	C	H	C	H		
Staphisine	82.83	9.94	83.38	9.34	83.48	9.56	82.36 82.62 82.85	9.47 9.73 9.77
“ dihydrochloride	73.96	9.17	74.40	8.63	74.85	8.86	73.84 73.70 74.37	9.08 8.94 9.04
“ dihydrobromide	65.43	8.11	65.77	7.63	66.47	7.87	64.95 64.97	7.92 7.84
“ monomethiodide	68.76	8.46	69.13	7.97	69.73	8.20	68.60	8.34
“ dimethiodide	59.17	7.45	59.41	7.03	60.24	7.26	58.98 58.40	7.41 7.29
Tetrahydrostaphisine	82.28	10.53	82.83	9.94	82.95	10.13	82.50 82.19	10.08 10.08

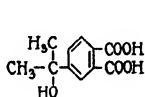
The calculated values for $C_{42}H_{58}ON_2$ are obvious by interpolation.

this carbon atom could afford an explanation for the formation of a 3-methylphenanthrene derivative on dehydrogenation. The C_{44} and C_{22} formulas which were originally suggested from the analytical data, but which are now less probable, would have permitted as an explanation for the formation of 3-methylretene the presence of the extra methyl group in the same position in the original C_{22} alkaloid. But this would require a departure from the usual diterpenoid structures. Studies are now in progress to establish, among other things, the correct formulations of this and related alkaloids.

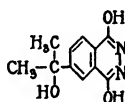
Parallel with the above work, studies have continued to determine also the identity of the previously recorded oxidation product of the $C_{19}H_{20}$

hydrocarbon obtained through the intermediate quinone, and which was tentatively interpreted as a "hydroxyisopropylphthalic acid, $C_{11}H_{12}O_6$ " (Formula VI). 1,2-Dimethyl-4-isopropylbenzene (7) was oxidized with permanganate to 4-hydroxyisopropylphthalic acid (Formula VI). As a check on its identity, the same acid was obtained also by oxidation of 2-aceto-1-methyl-4-isopropylbenzene (8) and by oxidation of 2-methyl-4-isopropylbenzoic acid (9). Its phthalic acid character was confirmed by the condensation of its dimethyl ester with hydrazine to the phthalazine derivative, 6-hydroxyisopropyl-1,4-dihydroxyphthalazine (Formula VII). The oxidation of the synthetic acid with nitric acid to trimellitic acid was in accord with a 4 position for the hydroxyisopropyl side chain.

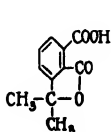
The synthetic $C_{11}H_{12}O_6$ acid appeared definitely different in properties from the acid from staphisine. Although the melting points of the two substances were similar (168–170°), a mixture showed some depression (163–165°). The alkaloid acid, after melting, resolidified with transfor-



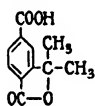
VI



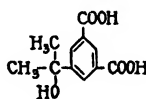
VII



VIII



IX



X

mation to high melting crystalline material (290°), while the synthetic acid after melting gave no evidence of the formation of such a substance. It seemed rather to form an ester type of polymer. Although the absorption spectra of the synthetic acid and that from staphisine are not identical (Fig. 1), there is sufficient similarity to suggest a general relationship, if not the unlikely possibility that the natural acid may have been a mixture which contained some 4-hydroxyisopropylphthalic acid. The exact identity of this substance is therefore still undetermined and the very limited amount of material which has been available has prevented its further study.

Consideration has also been given to the possibility that the so called " $C_{11}H_{12}O_6$ acid" could have been a position isomer, since there are six possible hydroxyisopropylbenzenedicarboxylic acids. Of these, four possess the hydroxyisopropyl residue in a position ortho to a carboxyl group and should not be sufficiently stable for isolation because of spontaneous lactonization. Hence the " $C_{11}H_{12}O_6$ acid" should not be one of these acids.

A check, however, on such expected lactonization was made by the synthesis and study of the behavior in the case of *3-hydroxyisopropylphthalic acid*. As expected, this substance could be isolated only as its corresponding *phthalide*, *3,3-dimethyl-7-carboxyphthalide* (Formula VIII). The latter was synthesized by permanganate oxidation of *1,2-dimethyl-3-hydroxyisopropylbenzene*, which was prepared by the reaction of the magnesium compound from *1,2-dimethyl-3-bromobenzene* with acetone. The lactone of hydroxyisopropylterephthalic acid (Formula IX) has already been studied by Cannizzaro and Gucci (10), who found that the dibasic acid itself can exist only in the form of salts in alkaline solution.

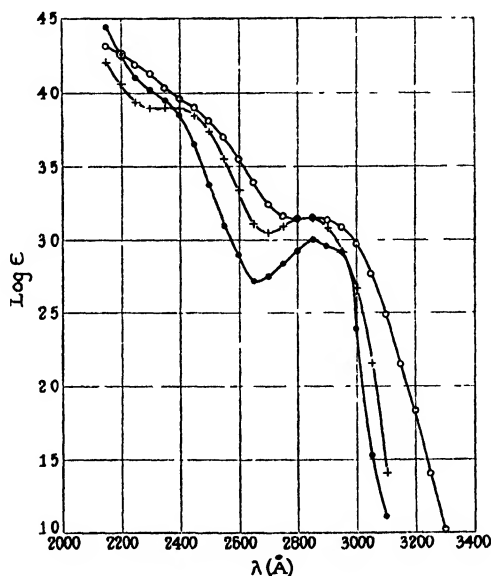


FIG. 1. ●, 5-hydroxyisopropylisophthalic acid; +, 4-hydroxyisopropylphthalic acid; ○, " $C_{11}H_{12}O_6$ acid" from staphisine.

A hydroxyisopropylidicarboxylic acid which can exist as such is *5-hydroxyisopropylisophthalic acid* (Formula X). This substance was prepared by oxidation of 5-isopropylisophthalic acid. It proved to be different from the so called " $C_{11}H_{12}O_6$ acid" (m.p. 300–305°). Its absorption spectrum is recorded in Fig. 1.

EXPERIMENTAL

2-(6-Isopropyl-2-naphthoyl)isobutyric Acid—A mixture of 30 gm. of 2-isopropyl-naphthalene (11) and 15 gm. of methylsuccinic anhydride was added to a cold solution of 34 gm. of aluminum chloride in 130 cc. of nitro-

benzene. After 12 hours, dilute HCl was added and nitrobenzene and excess isopropyl-naphthalene were removed with steam. The residual tar was dissolved in ether and the acids were extracted with Na_2CO_3 solution. The latter was reacidified and extracted with ether. The ether yielded crystalline material on evaporation. After two recrystallizations from ethanol, 8 gm. of the acid were obtained as needles and melted at $175-176^\circ$.

$\text{C}_{18}\text{H}_{20}\text{O}_4$. Calculated, C 76.01, H 7.09; found, C 76.25, H 6.66

The *methyl ester* was prepared by refluxing the acid for 12 hours with 3 per cent HCl in methanol. The yield from 7.5 gm. was 6.5 gm., m.p. $92-94^\circ$.

$\text{C}_{19}\text{H}_{22}\text{O}_4$. Calculated, OCH_3 10.40; found, OCH_3 9.95

1,3-Dimethyl-3-(6-isopropyl-2-naphthyl)- Δ^2 -butenoic Acid—A Grignard solution prepared from 0.77 gm. of magnesium and 2 cc. of methyl iodide was added dropwise to a solution of 6.43 gm. of the above methyl ester in 25 cc. of dry benzene. The mixture was refluxed for 3 hours and then decomposed with dilute H_2SO_4 . The benzene solution was extracted with Na_2CO_3 solution which, when reacidified, yielded a waxy solid. The collected material crystallized from ethanol, but repeated recrystallizations were found necessary to obtain the acid ash-free. The yield was 1.5 gm., and the melting point $185-186^\circ$.

$\text{C}_{19}\text{H}_{22}\text{O}_2$. Calculated, C 80.80, H 7.86; found, C 80.75, H 8.00

1,3-Dimethyl-7-isopropylphenanthrene—1 gm. of 3-(6-isopropyl-2-naphthyl)-1,3-dimethyl- Δ^2 -butenoic acid was refluxed for 4 hours with 1 gm. of red phosphorus in 5 cc. of HI (sp. gr. 1.7). The resulting oil was extracted with ether, and the extract was shaken successively with sodium bisulfite and NaOH solutions. The NaOH solution was acidified and re-extracted with ether. The latter, after concentration, yielded a residue of 250 mg. of crude 2-(6-isopropyl-2-naphthyl)isobutyric acid. This material without further purification was cyclized by heating with 1.5 cc. of 66 per cent H_2SO_4 on the steam bath for 1 hour. The mixture was diluted with water and extracted with ether. The ether extract was washed with dilute alkali and then yielded 150 mg. of residue, which consisted essentially of 4-oxo-1,3-dimethyl-7-isopropyl-1,2,3,4-tetrahydrophenanthrene. The crude ketone was refluxed for 12 hours with 75 mg. of amalgamated zinc in 2 cc. of HCl (sp. gr. 1.19). The crude tetrahydrophenanthrene derivative (125 mg.) isolated by ether extraction was heated with 190 mg. of selenium at $300-340^\circ$ for 24 hours. The dehydrogenation mixture was extracted with ether and the crude hydrocarbon which was recovered was dissolved in petroleum ether. This solution was passed through a 12 mm. \times 8 cm. column of activated Al_2O_3 (Merck and Company, Inc.). The column was developed in ultraviolet light with 1 per cent benzene in petroleum ether. The main fluorescing band was separately collected and yielded 100 mg. of

an oil which crystallized from ethanol. The yield of 1,3-dimethyl-7-isopropylphenanthrene was 70 mg. It formed diamond-shaped plates which melted at 73–76°.

$C_{19}H_{20}$. Calculated, C 91.88, H 8.12; found, C 91.91, H 8.02

The $C_{19}H_{20}$ hydrocarbon from staphisine was indistinguishable from the synthetic substance. Although its melting point was 70–72°, the mixture of the two substances melted at 70–75°.

The *picrate* of the synthetic product was obtained as yellow needles from ethanol and melted at 145–146°.

$C_{19}H_{20} \cdot C_6H_3O_7N_3$. Calculated, C 62.87, H 4.86; found, C 62.94, H 4.60

A melting point of the mixture of natural and synthetic picrates showed no depression (144–145°).

The 1,3,5-trinitrobenzene derivatives of both the natural and synthetic hydrocarbons were prepared. After recrystallization from ethanol, yellow needles were obtained from both sources, melting point 159–160°. The melting point of a mixture of the two derivatives was 159–160°. Because of the small amount of material available, this derivative was not analyzed.

Ethyl-(6-isopropyl-2-naphthyl) Ketone—Propionylchloride (37.5 cc.) was gradually added with cooling to a solution of 74 gm. of 2-isopropyl-naphthalene and 137 gm. of aluminum chloride in 250 cc. of nitrobenzene. After 2 days the mixture was decomposed with dilute HCl and the nitrobenzene was removed with steam. The residual oil was distilled at 1 mm. and yielded a crystalline distillate which was recrystallized from petroleum ether. The yield was 23 gm. and the melting point 72–74°.

$C_{18}H_{18}O$. Calculated, C 84.90, H 8.02; found, C 85.23, H 8.16

6-Isopropyl-2-naphthoic Acid; (a) *From 2-(6-Isopropyl-2-naphthoyl)isobutyric Acid*—0.89 gm. of the isobutyric acid derivative was heated on the steam bath with a solution of 50 gm. of potassium ferricyanide and 11 gm. of KOH in 75 cc. of water. After 24 hours an additional 25 gm. of ferricyanide and 5 gm. of KOH were added to the mixture and the heating was continued for another day. The resulting solution was acidified and then extracted with ether. The residue which remained after evaporation of the ether was sublimed at 0.03 mm. from a bath at 130°. The sublimate on recrystallization from dilute methanol yielded 0.3 gm. of the 6-isopropyl-2-naphthoic acid, which melted at 183–185°. Haworth (6) reported for his substance a melting point of 182–183°, but no analysis was given. The analysis of our substance proved to be unsatisfactory until purification over the methyl ester was accomplished. The latter after saponification yielded the acid which then melted at 192–194°.

$C_{18}H_{18}O_2$. Calculated, C 78.46, H 6.59; found, C 78.02, H 6.35

The *methyl ester* melted at 68–69° (Haworth reported 69–70°).

$C_{11}H_{10}O_2$. Calculated, C 78.90, H 7.07; found, C 78.90, H 7.09

(b) From *Ethyl-(6-methyl-2-naphthyl) Ketone*—1 gm. of the ketone was refluxed for 2 days with a sodium hypochlorite solution which contained an equivalent of 1 gm. of chlorine. The sodium salt of V formed a flocculent mass of fine crystals as the oxidation proceeded. After collection, it was decomposed with acid and the liberated acid was purified as described above. 0.8 gm. was obtained, which melted at 190–191°.

Found, C 78.48, H 6.62

l-Hydroxyisopropylphthalic Acid—1 gm. of 1,2-dimethyl-4-isopropylbenzene, prepared according to Klages (7), was dissolved in a mixture of 7 cc. of pyridine and 2 cc. of water. The solution was stirred on the steam bath during the gradual addition (over 1 hour) of 5 gm. of potassium permanganate. After reduction of all the reagent, the pyridine was removed with steam and the MnO_2 was filtered off. An additional 2 gm. of permanganate were then added and the oxidation was completed by heating for another hour. Excess reagent was destroyed with sodium bisulfite. The filtrate was treated with acid until only slightly basic and then concentrated *in vacuo* to a few cc. The concentrate was strongly acidified and extracted repeatedly with ether. The extract on concentration yielded a syrup which crystallized from a small volume of a mixture of acetone and ether. The yield was 0.7 gm. and the melting point 145–150°. After two recrystallizations from ethyl acetate-petroleum ether mixture and three from water, the melting point remained constant at 168–170° after preliminary softening at 163°. When heated further, no resolidification was noted. The acid from the staphisine hydrocarbon melted at 170°, but on raising the temperature crystallization occurred with subsequent melting at 290–294° (4). The melting point of a mixture of the synthetic acid with the latter was 162–165° and a small amount of the 290° melting material appeared in the melt.

$C_{11}H_{12}O_5$. Calculated, C 58.90, H 5.40; found, C 58.70, H 5.37

The same acid was produced in comparable yields by the similar oxidation of 2-aceto-1-methyl-4-isopropylbenzene (8) and 1-methyl-1-isopropylbenzoic acid* (9), melting point 168–170°.

Found, C 58.48, H 5.03

0.5 gm. of 4-hydroxyisopropylphthalic acid, when heated in a sublimation apparatus, melted at 170° with immediate evolution of water. The bath temperature was slowly raised to 200°. An appreciable amount of

* The latter substance was kindly supplied us by Dr. C. T. Lester, Emory University.

substance sublimed only when the pressure was reduced to 0.05 mm. and the temperature was raised to 250°. 80 mg. of a resin were collected. When the temperature was raised to 310°, about 20 mg. of additional material were distilled. The major portion of the substance remained undistilled as an amber vitreous residue.

Dehydration was also attempted by refluxing the phthalic acid with acetic anhydride, but again none of the substance with a melting point of 290° obtained from the alkaloid acid could be isolated.

6-Hydroxyisopropyl-1,4-dihydroxyphthalazine—The methylester was prepared from 0.2 gm. of 4-hydroxyisopropylphthalic acid with diazomethane. The oil obtained was heated at 100° with 0.2 gm. of hydrazine hydrate and 0.5 cc. of ethanol for 5 hours. The reaction product was dissolved in a small amount of water and carefully acidified with acetic acid. Two recrystallizations from ethanol yielded 0.1 gm. of fine needles; m.p. 220–225°.

$C_{11}H_{12}O_4N_2$. Calculated. C 59.97, H 5.50, N 12.73
Found. " 60.08, " 5.46, " 12.86

Trimellitic Acid from 4-Hydroxyisopropylphthalic Acid—0.1 gm. of 4-hydroxyisopropylphthalic acid was heated on the steam bath with 2 cc. of HNO_3 (sp. gr. 1.5) and a drop of 50 per cent manganous nitrate solution and then evaporated to dryness. The residue was collected with HNO_3 and recrystallized twice from water. Because of its solubility in H_2O , only 10 mg. of the recrystallized acid resulted. It melted at 220–235°. An authentic sample of trimellitic acid melted at 220–235° and a mixture of the two substances showed no depression.

1,2-Dimethyl-3-hydroxyisopropylbenzene—To a solution of the Grignard reagent prepared from 11.5 gm. of 1,2-dimethyl-3-bromobenzene (12) and 1.5 gm. of magnesium, 5.5 cc. of acetone in 25 cc. of ether were gradually added. After standing overnight, the mixture was decomposed with dilute HCl and the ether removed. 9 gm. of an oil resulted, which partially crystallized after standing at 0°. After two recrystallizations from petroleum ether, the yield of the carbinol was 1 gm. and the m.p. 67–68°.

$C_{11}H_{14}O$. Calculated. C 80.43, H 9.82
Found. " 80.35, " 9.62

3,3-Dimethyl-7-carboxyphthalide—0.16 gm. of 1,2-dimethyl-3-hydroxyisopropylbenzene was oxidized with 0.65 gm. of permanganate, as described above for 1,2-dimethyl-4-isopropylbenzene. When the alkaline filtrate from the reaction mixture was acidified, an immediate crystalline precipitate appeared. After two recrystallizations from water, 50 mg. of substance were obtained which melted at 187–188°.

$C_{11}H_{10}O_4$. Calculated, C 64.05, H 4.89; found, C 63.77, H 4.80
Neutralization equivalent. Calculated, 206; found, 212

3 gm. of α -naphthyltrimethylcarbinol (13) were oxidized with 14.5 gm. of permanganate. 0.5 gm. of a crude acid was obtained, which melted at 120–135°. After four recrystallizations from an ethyl acetate-petroleum ether mixture, the melting point was gradually raised to 188–189° and, when mixed with the above substance, showed no depression.

5-Hydroxyisopropylisophthalic Acid—0.5 gm. of 5-isopropylisophthalic acid, prepared according to Doebner (14), was similarly oxidized with 0.6 gm. of permanganate. 100 mg. of a crude acid resulted, from which 60 mg. of product were obtained after two recrystallizations from water; m.p. 300–305°.

$C_{11}H_{12}O_6$. Calculated, C 58.90, H 5.40; found, C 58.70, H 5.30
Neutralization equivalent. Calculated, 112; found, 113

SUMMARY

The hydrocarbon $C_{19}H_{20}$ previously obtained on dehydrogenation of staphisine has been identified as 1,3-dimethyl-7-isopropylphenanthrene, and suggests the possible diterpenoid character of the original molecule.

The formulation of staphisine has been revised to the possible dimolecular formula $C_{42}H_{40}ON_2$, which can be composed of two diterpenoid C_{20} bases which contain in addition N-methyl groups.

The results of synthetic studies contradict the interpretation originally suggested for the "hydroxyisopropylphthalic acid, $C_{11}H_{12}O_6$," which had been obtained on oxidation of this $C_{19}H_{20}$ hydrocarbon from staphisine.

All microanalyses were performed by Mr. D. Rigakos of this laboratory.

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LETTERS TO THE EDITORS

THE ISOLATION OF Δ^9 -ETIOCHOLENOL-3(α)-ONE-17 FROM HUMAN URINE

Sirs:

The isolation from urine of normal and of diseased individuals of two steroids containing an oxygen atom at the 11 position, namely, androstane-diol-3(α),11(β)-one-17 (I) and etiocholanol-3(α)-dione-11,17 (II), has been previously reported.¹

We have recently isolated Δ^9 -etiocholenol-3(α)-one-17 (III) from the neutral ketonic fraction of acid-hydrolyzed urine of a patient with cancer of the breast. Its presence has been established also in the urine of certain patients with cancer of the prostate, lymphatic leucemia, essential hypertension, and Cushing's syndrome. It has not been detected in the individual urine collections of twelve normal men and eight normal women, nor in large pools of urine from both sexes. It was present in the urine of one woman, aged 76 years, and of one man, aged 72 years, both of whom were apparently in good health.

This compound after several recrystallizations from acetone melted at 169–170°; $[\alpha]_D^{22} = +151^\circ \pm 4.5^\circ$ (2.25 mg. in 2.00 ml. of acetone). When mixed with an authentic sample of Δ^9 -etiocholenol-3(α)-one-17, obtained in the degradation of etiocholanol-3(α)-dione-11,17² (m.p. 171°, corrected; $[\alpha]_D = +155^\circ \pm 2^\circ$ (acetone)), the mixture melted at 168–170°. The two samples exhibited identical infra-red absorption spectra. When the compound isolated from urine was treated with perbenzoic acid in chloroform solution at 0° for 48 hours, an epoxide was obtained which melted at 177–178.5°; $[\alpha]_D^{23} = +119^\circ \pm 13^\circ$ (1.51 mg. in 2.00 ml. of ethanol), and which was identical with the epoxide prepared in a similar fashion from the synthetic sample (m.p. 175–177°; $[\alpha]_D^{23} = +123^\circ \pm 6.5^\circ$ (3.08 mg. in 2.00 ml. of ethanol)). The melting point of a mixture of these two epoxides showed no depression and their infra-red absorption spectra were identical.

¹ Mason, H. L., and Kepler, E. J., *J. Biol. Chem.*, **161**, 235 (1945). Lieberman, S., and Dobriner, K., *J. Biol. Chem.*, **166**, 773 (1946).

² Sarett, L. H., *J. Biol. Chem.*, **162**, 619 (1946).

The Δ^9 -etiocholenol-3(α)-one-17 is probably produced from etiocholane-diol-3(α),11(β)-one-17 (IV) by dehydration during the acid hydrolysis of the urine, since it is known that the 11- β -hydroxyl group is removed easily by treatment with acids.³ An attempt is under way to isolate IV.

It is to be noted that except in two instances Δ^9 -etiocholenol-3(α)-one-17 has been obtained only from the urine of individuals with disease. Whereas androstanediol-3(α),11(β)-one-17 is a normal metabolite, its diastereoisomer, etiocholanol-3(α),11(β)-one-17, the probable precursor of III, presumably is present principally in the urine of individuals with disease. This fact suggests that IV is a product of deranged metabolism of adrenal cortical hormones or a metabolite of an abnormal precursor.

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³ Shoppee, C. W., and Reichstein, T., *Helv chim acta*, **26**, 1316 (1943).

RESULTS OF ADMINISTRATION OF ANTERIOR PITUITARY ADRENOCORTICOTROPIC HORMONE TO A NORMAL HUMAN BEING

Sirs:

We have administered anterior pituitary adrenocorticotrophic hormone to a young woman maintained on a constant diet. The hormone was injected subcutaneously in five equal portions between 7.30 a.m. and 11.30 p.m. The total daily dose was 25 mg. for 6 days, 50 mg. for 6 days, and 100 mg. for 11½ days. During the first and second periods the only significant change was in the excretion of cortin-like substances; this excretion increased from an average control value of 0.180 to 0.255 and 0.387 mg. per day, respectively. When 100 mg. were administered daily, the cortin-like substances excreted in the urine increased to 1.44 mg. on the 10th day and the 17-ketosteroids excreted in the urine increased from an average control value of 4.8 to 15.5 mg. on the 10th and 11th days. The values for the 17-ketosteroids and cortin-like substances excreted in the urine quickly reverted to control levels after withdrawal of the hormone.

There were also a slightly increased excretion of nitrogen, a decreased excretion of creatine, and a pronounced decrease in hemoglobin. There were no demonstrable changes in the excretion of sodium, potassium, chloride, phosphorus, and creatinine. Pregnanediol could not be demonstrated in the urine in the first part of the menstrual cycle, but it was present in the latter part of the cycle. The experimental period did not differ from the control period in this respect. There were no significant changes in the excretion of estrogens and gonadotropic hormone. The content of sodium, potassium, chloride, ascorbic acid, protein, non-protein, nitrogen, and inorganic phosphorus in the blood remained essentially unchanged. Changes in the lymphocyte count, carbon dioxide-combining power, and cholesterol were of questionable significance. The electrophoretic pattern of the plasma remained unchanged during the entire period of study, and there was no change in the alkaline phosphatase activity.

Isolation of the urinary steroids did not reveal any qualitative change in pattern. Androsterone, etiocholan-3(α)-ol-17-one, pregnane-3(α),20(α)-diol, and cholesterol were isolated from urine collected before and during administration of the hormone. A small amount of a ketone melting between 238–240° was isolated from the control urine (72 days). The hope that 11-hydroxyandrosterone, as a metabolite of adrenal steroids, would appear or be increased in the urine during administration of the hormone was not realized.

The increased excretion of steroids in the urine is considered to be defi-

nite evidence of stimulation of the adrenal cortex by the pituitary hormone. The increase in the excretion of nitrogen during the last period was probably significant, but was not great enough, in comparison with some of the control data, to be given much weight. The significance of the decrease in the excretion of creatine and of the decrease in hemoglobin is not evident. The latter condition is the opposite of what is encountered in cases of Cushing's syndrome, in which there is often a demonstrable hyperfunctioning lesion of the adrenal cortex.

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ENZYMATIC HYDROLYSIS OF ACETYLDEHYDROALANINE

Sirs:

Glycyldehydroalanine (glycyl- α -aminoacrylic acid) is rapidly hydrolyzed to glycine, ammonia, and pyruvic acid in aqueous extracts of all animal tissues studied, in all species investigated.^{1,2} Chloroacetyldehydroalanine, on the other hand, is hydrolyzed with appreciable velocity in extracts only of kidney, liver, and pancreas. In the case of both substrates, ammonia and pyruvic acid appear in equivalent amounts.¹

A still simpler representative of this class of compounds, namely acetyldehydroalanine (N-acetyl- α -aminoacrylic acid), is hydrolyzed in aqueous

*Ammonia and Pyruvic Acid Derived from Acetyldehydroalanine in Aqueous Extracts of Rat Tissues**

Tissue†	Ammonia‡	Pyruvic acid‡	Molar ratio of ammonia to pyruvic acid
Kidney	178	1278	0.9
Liver	64	462	0.9

* The digests consisted in 1 cc. of aqueous extract equivalent to 333 mg. of tissue plus 2 cc. of 0.2 M borate buffer at pH 8.15, plus 1 cc. of either water or 0.025 M peptide. The peptide solution was neutralized with dilute NaOH before use and was stable for many weeks. The initial and final pH of the digests was close to 8.0. Tissue extracts were dialyzed 4 hours against distilled water at 5° before use. The incubation period was 4 hours at 37°. Values corrected for extract blanks. Results negative with heated extracts.

† Extracts of spleen, brain, pancreas, and muscle showed little or no activity.

‡ In terms of micrograms. Theory for ammonia 350; for pyruvic acid 2200.

extracts of rat kidney and liver (see the table). As in the case of the glycyl and chloroacetyl compounds, the nearly equivalent amounts of ammonia and pyruvic acid which appear, combined with the fact that glycine amide is split very slowly and chloroacetamide and acetamide not at all, indicate that the dehydropeptides are hydrolyzed almost entirely at the peptide bond. Under conditions whereby acetyldehydroalanine is readily hydrolyzed in kidney extracts, acetyldehydrophenylalanine (N-acetyl- α -aminocinnamic acid) is not affected.

Acetyldehydroalanine in aqueous solution, like other dehydropeptides, gives a characteristic absorption spectrum in the ultraviolet.² When

¹ Greenstein, J. P., and Leuthardt, F. M., *J. Nat. Cancer Inst.*, **5**, 209, 223, 249 (1945); **6**, 197 (1946).

² Carter, C. E., and Greenstein, J. P., *J. Nat. Cancer Inst.*, **7**, 51 (1946).

dehydropeptidase activity of rat kidney extracts is measured spectrophotometrically by the rate of decrease in absorption of the digest at 2500 A, and expressed in terms of $\text{mg.} \times 10^{-2}$ of substrate split per hour per mg. of total N in the extract,² the values for each of the substrates are, respectively, glycyldehydroalanine 820, chloroacetyldehydroalanine 53, and acetyldehydroalanine 13. Compared with their saturated analogues, the susceptibility of the dehydropeptides to intracellular hydrolysis is considerable, and suggests that this class of unsaturated compounds may play an important rôle in certain phases of protein metabolism.

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THE VACCENIC ACID CONTENT OF VARIOUS FATS AND OILS*

Sirs:

Vaccenic acid (Δ -11,12-elaidic acid) has recently been reported by Boer, Jansen, and Kentie¹ to possess growth-stimulating properties. These authors found that the growth rate of rats was more rapid when butter fat was in the ration than when rape-seed oil was used. The addition of vaccenic acid to the latter diet at a level of 0.1 per cent of the total ration caused the rate of growth to approximate that of the animals fed butter fat. From this it was concluded that this acid was one of the compounds in butter fat responsible for its superior nutritive value as compared with vegetable oils.

Fat	Per-cent vaccenic acid		
	Bertram ²	Grossfeld and Simmer ³	Present study
Butter fat	0.01	1.13-4.69	0.50-0.70
Lard		0.20	0.071-0.127
Mutton fat	0.96		0.16-0.195
Margarine	0.27	1.49	
Beef fat			0.16-0.213
Corn oil			0.0
Cottonseed oil			0.0
Soy bean oil			0.0
Coconut "			0.0
Rat fat			
" fed butter fat			0.08
" " corn oil			0.0
" " " " + vaccenic acid			Trace

* These values were obtained by calculation from the iodine number after a lead soap separation. 2.5 gm. samples were used. We have carried out similar analyses; however, the results have always been high, especially in the case of the vegetable oils.

Since the data in the literature on the occurrence of vaccenic acid are entirely restricted to fats of animal origin, various animal fats and vegetable oils were analyzed for this compound according to the method of Bertram.²

* This work was aided by grants from the National Dairy Council, Chicago, in behalf of the American Dairy Association, by the Evaporated Milk Association, Chicago, and the Wisconsin Alumni Research Foundation.

¹ Boer, J., Jansen, B. C. P., and Kentie, A., *Nature*, **158**, 201 (1946).

² Bertram, S. H., *Biochem. Z.*, **197**, 433 (1928).

³ Grossfeld, J., and Simmer, A., *Z. Untersuch. Lebensmittel*, **59**, 237 (1930).

Briefly the method used consisted of separating the fatty acids obtained from a 500 gm. sample of the oil by means of the lead soap procedure in order to remove most of the unsaturated acids other than vaccenic. The solid fraction was converted to the free acids, and most of the saturated acids were then removed by use of the mercury salt separation. After removal of the mercury, the vaccenic acid was recrystallized twice from acetone. It melted at 34.5–36° (Bertram reports 39°) and had an iodine number of 83.2 (calculated 89.9). The results are given in the table together with those reported by other investigators. Also included in the table are data secured on the pooled fat of rats fed a synthetic diet plus butter fat, as well as from rats fed a synthetic diet plus corn oil and the latter diet plus vaccenic acid.

Because of solubility influences of the soluble lead soaps and the possible occurrence of unsaturated acids other than vaccenic which might possess chemical properties similar to the saturated acids, the results given are not necessarily quantitative. Traces of vaccenic acid would probably escape detection by this procedure. It is of interest that the highest amounts of vaccenic acid have been found in fats obtained from ruminants.

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OLEIC ACID AS A GROWTH FACTOR FOR VARIOUS LACTOBACILLI

Sirs:

In a further investigation of the nutritional requirements of the lactic acid bacteria, it was noted that certain organisms failed to grow on a modified Tepley and Elvehjem medium.¹ The modified medium contained no alanine or norited peptone; the sodium citrate was replaced with sodium acetate and the medium supplemented with 10 γ of inositol, 10 γ of choline, 2 γ of pimelic acid, 100 γ of thymine, 10 γ of thiamine, 11 γ of *p*-amino-benzoic acid, and 0.2 γ of pteroylglutamic acid per 10 ml. of medium.

Requirement of Various Lactobacilli for Sodium Oleate

The results are expressed in ml. of 0.1 N acid.

Organism	Incubation period	Basal + sodium oleate		Basal + 75 mg yeast extract + sodium oleate (γ per 10 ml)					
		None	100 γ per 10 ml.	0	10	50	100	500	4000
<i>L. acidophilus</i> , 332	6 days	1 0	1 4						
	48 hrs.			2 8	3 5		9 7	10 4	10 2
" " 832	6 days	1 0	0 8						
	75 hrs.			3 8	5.6		8.2	7 2	9 0
" <i>plantarum</i> , 4940	6 days	1 1	0 5						
	71 hrs.			2 8	3 7	6 5	8 0	5 1	0 3
" <i>acidophilus</i> , 4357	6 days	0 6	0 4						
	18 hrs.			3 4	4 1	6.3	8 9	10 2	10.5
" <i>leichmannii</i> , 4797	6 days	0 7	0.7						
	20 hrs.			1.9	5 0	9 2	9 4	3 2	0 8
" <i>acidophilus</i> , 4355	6 days	0 6	0.5						
	48 hrs.			4 2	5 1	6 0	7 1	9 2	8 0
" <i>delbruckii</i> , 9649	6 days	0.6	0.5						
	48 hrs.			5.5	8.2	9.9	10 0	10.8	10.0

When the medium was further fortified with 75 mg. per tube of yeast extract (Difco), slight growth was obtained on prolonged incubation. On serial subculture the growth of *Lactobacillus leichmannii* and *Lactobacillus delbrückii* decreased to maximum, indicating that adaptation to the medium was taking place. With all other cultures only the slight growth noted in the first transfer persisted in the serial transplants. The particular lot of yeast extract was chosen because of its content of strepogenin. The amount of strepogenin present in 75 mg. of the yeast extract was equivalent to 13 times the amount necessary to produce half maximum stimula-

¹ Tepley, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, **157**, 303 (1945).

tion of *Lactobacillus casei* under the defined conditions of assaying for streptogenin.²

The addition of sodium oleate to the medium containing yeast extract allowed for maximum growth. The results are presented in the table. The amount of sodium oleate necessary to produce maximum growth varies with each organism. *Lactobacillus plantarum* and *Lactobacillus leichmannii* exhibited toxicity to increasing amounts of sodium oleate.

To demonstrate the stimulatory nature of sodium oleate for *Lactobacillus leichmannii*, the assay must be read after an incubation period of from 16 to 20 hours.

The various lactobacilli listed in the table can be successfully subcultured on this medium.

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² Sprince, H., and Woolley, D. W., *J. Exp. Med.*, **80**, 213 (1944).

A FACTOR IN PLASMA WHICH ACCELERATES THE ACTIVATION OF PROTHROMBIN*

Sirs:

The explanation for differences in activation rates¹ between plasma prothrombin and purified prothrombin fractions is furnished by the discovery of an accelerator factor in plasma which, when mixed with plasma prothrombin fractions, permits rapid activation of prothrombin by thromboplastin and calcium ions. The accelerator is adsorbed on $\text{Mg}(\text{OH})_2$, is water-soluble, is less soluble in concentrated $(\text{NH}_4)_2\text{SO}_4$ solution than prothrombin, and can be obtained from bovine plasma after storage at 8° for 18 days. The factor is not fibrinolysin. It is not one of the hypothetical prothrombins postulated by Quick.²

To obtain the accelerator, prothrombin Product 7 is first prepared exactly as described.³ Next, at 0° , saturated $(\text{NH}_4)_2\text{SO}_4$ is added. At 30 per cent of saturation the precipitate is discarded. The supernatant fluid is brought to 40 per cent of saturation. The precipitate is washed several times with cold 40 per cent saturated $(\text{NH}_4)_2\text{SO}_4$, dissolved, dialyzed against water, and dried from the frozen state. To test the activity of this fraction the two-stage method of prothrombin analysis was used.⁴ Some representative data are presented (see the table).

Plasma fractions	Minimum time for maximum thrombin formation
Prothrombin 470314	10 min.
Same + accelerator fraction	3 "
Prothrombin 470324	28 "
Same + accelerator fraction	7 "
Prothrombin 470325	3 hrs
Same + accelerator fraction	10 min.

These facts provide direct evidence that changes in the activation rate of prothrombin are a part of the physiological mechanism concerned in blood clotting. Furthermore, variations in accelerator concentration can

* Aided by a grant from the National Institute of Health.

¹ Seegers, W. H., Brinkhous, K. M., Smith, H. P., and Warner, E. D., *J. Biol. Chem.*, **126**, 91 (1938).

² Quick, A. J., *Am. J. Physiol.*, **140**, 212 (1943).

³ Seegers, W. H., Loomis, E. C., and Vandenbelt, J. M., *Arch. Biochem.*, **6**, 85 (1945).

⁴ Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, **114**, 667 (1936).

cause a serious error in the one-stage method⁵ of analysis for prothrombin. Later we hope to discuss the relationship of our work to that of Fantl and Nance.⁶

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⁵ Quick, A. J., *Am. J. Clin. Path.*, **15**, 560 (1945).

⁶ Fantl, P., and Nance, M., *Nature*, **158**, 708 (1946).

FIXATION OF CARBON DIOXIDE BY A PLANT OXALACETATE CARBOXYLASE*

Sirs:

The presence of an oxalacetate carboxylase in parsley root has been reported previously from this laboratory.¹ The reaction catalyzed by this system has been found to be reversible, since it fixes $C^{14}O_2$ into oxalacetate during the decarboxylation of the latter in the presence of $NaHC^{14}O_3$.

The enzyme was prepared essentially as previously reported¹ and completely freed from pyruvic carboxylase by prolonged dialysis. The preparation was lyophilized and made up to the desired strength by dissolving in water. The decarboxylation reaction, measured in 0.1 M acetate buffer, pH 5.0, in the presence of 0.01 M $MnCl_2$, follows first order kinetics. The difference between the first order rate constants, k ($k = (2.303/t) \log (C_0/C)$), observed with active and with heat-inactivated enzyme, divided by mg. of enzyme preparation, may be used as a measure of activity. For the enzyme employed in the experiments reported, $k = 0.075 \text{ min.}^{-1}$ per mg.

The exchange experiments with oxalacetate were conducted by the procedures recently described.² The initial reaction mixture contained 1.2×10^{-2} M phosphate buffer, pH 6.0, 10^{-3} M $MnCl_2$, 4.25×10^{-2} M oxalacetate, 3.6×10^{-3} M $NaHCO_3$ containing C^{14} , and 180 mg. of enzyme in a total volume of 10 ml. The reaction was incubated at 30° for 8 minutes, at which time approximately half of the oxalacetate had been decarboxylated. The activity of the β -carboxyl carbon liberated by aniline citrate was at this time found to be 0.49 ± 0.02 per cent of the activity of the $NaHCO_3$ of the medium at the end of the incubation period. The control run with the heat-inactivated enzyme showed no detectable fixation after 30 minutes incubation.

The enzyme preparation used in these experiments contains a malic dehydrogenase active with triphosphopyridine nucleotide. This activity can be demonstrated by measuring the reduction of 2,6-dichlorophenol indophenol. It can also be detected by measuring the reduction of cytochrome *c* in the presence of cytochrome reductase and TPN. In a typical experiment of the latter type, the reaction mixture initially contained 3.7×10^{-3} M glycylglycine, pH 7.4, 3.7×10^{-4} M $MnCl_2$, 1.65×10^{-5} M TPN,

* Aided in part by grants from the John and Mary R. Markle Foundation and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

¹ Vennesland, B., and Felsner, R. Z., *Arch. Biochem.*, **11**, 279 (1946).

² Wood, H. G., Vennesland, B., and Evans, E. A., Jr., *J. Biol. Chem.*, **159**, 153 (1945). Utter, M. F., and Wood H. G., *J. Biol. Chem.*, **164**, 455 (1946).

500 γ of cytochrome reductase, 4.59×10^{-8} M cytochrome *c*, 5.5×10^{-4} M L-malic acid, and 1.5 mg. of enzyme in a total volume of 1.35 ml. At 26°, the specific reaction rate constant for the first order reduction (per mg. of enzyme used) was 0.038 min.⁻¹ corrected for the control without malic acid.

In accordance with expectations, the parsley root preparation was found to be capable of fixing CO₂ in the malate fraction when pyruvate and malate were incubated with C¹⁴O₂. There is no oxidation-reduction between pyruvate and malate, since the preparation contains no lactic dehydrogenase. Fumarase is likewise absent.

These results suggest that the synthesis of the plant dicarboxylic acids may occur by way of an initial Wood-Werkman reaction, as has been found to be the case with bacteria and animal tissues.³

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³ Evans, E. A., Jr., Vennesland, B., and Slotin, L., *J. Biol. Chem.*, **147**, 771 (1943). Moulder, J. W., Vennesland, B., and Evans, E. A., Jr., *J. Biol. Chem.*, **160**, 305 (1945). Ochoa, S., Mehler, A., and Kornberg, A., *J. Biol. Chem.*, **167**, 871 (1947). Wood, H. G., *Physiol. Rev.*, **26**, 198 (1946).

THE PORPHYRIN PRODUCED BY DIPHTHERIA BACILLUS

Sirs:

A. M. Pappenheimer, Jr., has recently described the effect of iron on toxin and porphyrin production by *Corynebacterium diphtheriae*.¹ He assumed that the porphyrin produced by this organism was hematoporphyrin but recognized the need for final identification of the diphtherial porphyrin by isolation analysis.

We have isolated the porphyrin produced by the same strain of organism grown on the same medium. In ether its absorption maxima, as determined by a Hartridge reversion spectroscope, are at 622.5, 574.1, 528.9, and 496.9 (order of intensity IV > I > III > II). In 0.15 N HCl, the maxima were at 590.1 and 546.8 (II > I). The methyl ester of this porphyrin was readily soluble in chloroform and in benzene, and moderately soluble in methyl alcohol and in anhydrous ether. From this last solvent, the ester crystallized in thick truncated (oblate) needles with a melting point of 155–157° and remelted at 181–182°. Its spectral absorption curve determined with a Hilger photoelectric spectrophotometer was identical with that of coproporphyrin I (a pure specimen of coproporphyrin III was not available). The methyl ester yielded a copper complex melting at 218° and with absorption maxima in chloroform at 563 and 526 (I > II). Chromatography of the methyl ester in benzene through CaCO₃ or alumina revealed that the material behaved as an entity except for two small zones at the top of the column. These zones have not yet been identified.

These properties, especially the phenomenon of the double melting point, leave little doubt that the porphyrin produced by *Corynebacterium diphtheriae* is mainly coproporphyrin III.

We have isolated yields as high as 4 mg. of pure coproporphyrin III methyl ester per liter of diphtherial culture filtrate. These filtrates constitute, therefore, an important and hitherto unexplored source of coproporphyrin III, and details of a method whereby this material can be obtained as a by-product in the large scale production of purified diphtheria toxoid will be published shortly.

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¹ Pappenheimer, A. M., Jr., *J. Biol. Chem.*, **167**, 251 (1947).

RULES FOR THE NOMENCLATURE OF THE NATURAL AMINO ACIDS AND RELATED SUBSTANCES

The following rules are designed to eliminate the current confusion in the nomenclature of the α -amino acids that arise from the hydrolysis of proteins, and of the compounds and derivatives of these acids.

Rule 1. The configurational relationship of the asymmetric α -carbon atom of an amino acid capable of optical isomerism should be indicated by a symbol prefixed to the name; however, if a specific statement or the context makes it clear which isomer is under consideration, the symbol may be omitted.

Examples: Leucine may be named without prefix if the preparation mentioned is stated to be the optically active isomer derived from a protein source. Leucine may be named without prefix if the preparation mentioned is stated to be synthetic and not resolved and therefore the optically inactive mixture of isomers. Leucine may be named without prefix in a general statement that is true for either optical isomer or for any mixture of these.

Rule 2. Distinction between the stereoisomers of the amino acids is made by a prefixed *small capital letter* D or L to denote the configurational family to which the α -carbon atom belongs. The D and L are to be pronounced *dee* and *ell*, respectively, not *dextro* and *levo*. An additional symbol to denote the direction of the rotation (*i.e.*, a plus or a minus sign enclosed in parentheses) is not necessary.

Examples: L-leucine, D-valine, L-phenylalanine, L-threonine.

The optically inactive mixture or racemic compound of the stereoisomers is designated by the prefix DL- in *small capital letters*.

Examples: DL-leucine, DL-valine, DL-methionine.

Rule 3. Where confusion is possible between the use of the capital letter prefix for the configuration of the α -carbon atom in amino acid nomenclature and for that of the highest numbered asymmetric carbon atom in carbohydrate nomenclature, a subscript is added to the capital letter prefix. Where the prefix is used in the amino acid sense, the subscript s is added; where the prefix is used in the carbohydrate sense, the subscript g is added. These subscripts (lower-case roman letters) refer, respectively, to serine, the fundamental substance to which amino acids that bear structural resemblance to the carbohydrates can be formally related, and to glyceric aldehyde, the fundamental substance to which the configuration of the carbohydrates is formally related.

Examples: L₂-threonine for which the synonym in carbohydrate nomenclature is 2-amino-2,4-dideoxy-D₂-threonic acid, D₂-threonine for which the synonym is 2-amino-2,4-dideoxy-L₂-threonic acid, L₂-allothreonine for which the synonym is 2-amino-2,4-dideoxy-L₂-erythronic acid, D₂-allothreonine for which the synonym is 2-amino-2,4-dideoxy-D₂-erythronic acid. Papers that deal with both carbohydrates and amino acids and which make use of the prefixes should employ subscripts at least for the names of the amino acids. The acids derived from amino sugars should be named in conformance with carbohydrate nomenclature but with the use of the subscript, e.g. D₂-glucosaminic acid for 2-amino-2-desoxy-D₂-gluconic acid.

Rule 4. If the configurational relationship of the α -carbon atom has not been definitely established, the actual direction of the rotation in a specified solvent, preferably of the free amino acid in water, is designated by the prefixes *dextro* or *levo* in *lower-case italic letters* or, alternatively, by a plus or a minus sign enclosed in parentheses.

Examples: *dextro*hydroxytryptophan, (+)-hydroxytryptophan.

Rule 5. The prefix *meso-* or its abbreviation *ms-* in *lower-case italic letters* is used to denote the isomers of amino acids and derivatives of these that are optically inactive because of internal compensation.

Examples: *meso*-lanthionine, *ms*-cystine.

Rule 6. Where the amino acid has two centers of asymmetry so constituted that internal compensation is impossible, the form which has the L-configuration at the α -carbon atom is designated the L-amino acid and the form that has the opposite configuration at *both asymmetric carbon atoms* is designated the D-amino acid.

Examples: L-threonine, hydroxy-L-proline, L-isoleucine for the isomers derived from proteins, D-threonine, hydroxy-D-proline, D-isoleucine for their optical isomers.

The diastereoisomers of these substances are designated with the prefixes L-*allo-* and D-*allo-* joined directly to the name, the prefixed letter in each case denoting the configurational family of the α -carbon atom.

Examples: L-allothreonine, allosehydroxy-L-proline, L-alloisoleucine, D-allothreonine, allosehydroxy-D-proline, D-alloisoleucine.

Rule 7. Salts and derivatives of amino acids including peptides are designated with the use of a *small capital letter* to denote the configurational family of the α -carbon atom or atoms, this letter being placed immediately before the name of the parent acid or its radical. The customary rules of nomenclature are otherwise observed.

Examples: L-histidine monohydrochloride monohydrate, copper L-aspartate, D-ornithine dihydrochloride, acetyl-L-tryptophan, diethyl D-glutamate, 4-hydroxy-L-

proline, 3,5-diiodo-L-tyrosine, β -hydroxy-DL-glutamic acid, L-leucyl-L-valine, glycy-DL-leucine. For the purposes of this rule, phenylalanine is regarded as the name of the parent amino acid because of long established usage; thus N-benzoyl-L-phenylalanine, not N-benzoyl- β -phenyl-L-alanine. Names in which the prefixes involve amino acid configurations are treated similarly; thus, S(D- β -amino- β -carboxyethyl)-D-homocysteine for D-cystathionine.

Rule 8. Where a trivial name is applied to a compound that contains two α -amino carboxylic acid groupings attached to dissimilar carbon chains, the configurational designation is that of the α -carbon atom of the *smaller of the two chains*.

Examples: D-cystathionine for S(D- β -amino- β -carboxyethyl)-D-homocysteine, L-allo-cystathionine for S(L- β -amino- β -carboxyethyl)-D-homocysteine

However, the introduction of new trivial names should be avoided in the absence of compelling reasons.

Background of Rules

The term natural amino acids includes the α -amino acids that are liberated by the hydrolysis of proteins as well as a considerable number of amino acids and related substances found in plant and animal tissues as products of metabolism. By far the greater proportion of these substances belongs to the L system but, in recent years, an increasing number of observations has been made of the presence of amino acids of the opposite configuration, chiefly in products of the metabolism of lower organisms. Accordingly the term natural amino acid is no longer synonymous with the term L-amino acid.

The present rules deal chiefly with substances that are the concern of biochemists and physiologists. They are intended to solve certain of the problems of nomenclature in this field which have arisen because of the use in the literature of several different earlier systems for naming these substances. The origin of the present confusion is traced in the following paragraphs.

The early literature and such reference works as Abderhalden's *Handlexikon* and Beilstein name the amino acids with a prefix that shows the direction of optical rotation of an aqueous solution of the substance at a reaction in the neutral range. Thus, for example, for the stereoisomers that are obtained from proteins, the names *d*-alanine, *d*-valine and *l*-leucine were used.

Subsequently, a suggestion made by Wohl and Freudenberg (Ber., **56**, 309 (1923)) was widely adopted. According to this, the prefix was used only to denote the *configurational family* to which the α -carbon atom of the amino acid belongs, and the actual direction of rotation was indicated by a plus sign or a minus sign enclosed in parentheses. Thus, for the same

three substances, the names *l*(+)-alanine, *l*(+)-valine and *l*(-)-leucine were introduced.

In recent years, it has been demonstrated that, in general, the stereoisomers of the amino acids that are obtained from proteins belong to the *l* family with respect to the configuration of the α -carbon atom. As a result, these isomers are frequently spoken of as the *l*-amino acids and the individual substances mentioned above may appear as *l*-alanine, *l*-valine and *l*-leucine notwithstanding the fact that two of these names are different from the ones employed in the early literature.

Even those chemists who carefully adhere to the Wohl-Freudenberg system of nomenclature differ among themselves in usage because there is no generally accepted convention regarding the solvent to be employed for the observation of the direction of the rotation. Certain chemists consistently use the direction in neutral aqueous solution; others prefer to use the direction of rotation in the presence of an excess of mineral acid, in part because some of the amino acids are so insoluble in water at neutral reaction that observation of the rotation is difficult, and in part because strongly acid solutions contain only a single ionic species and the rotation is therefore not sensitive to moderate changes in the acid concentration. In most cases, both conventions with regard to the solvent lead to the same name, but there are six cases, cysteine, histidine, leucine, methionine, serine and tryptophan, where the water convention leads, for the isomers derived from proteins, to a name prefixed by *l*(-)-, while the acid convention leads to a name prefixed by *l*(+)-.

The name threonine provides a still more difficult problem. When this substance was first isolated from proteins and thoroughly characterized by Rose in 1936, it was named *d*(-)-threonine in order to draw attention to the configurational relationship with D-threose. Because of this derivation of the name, the prefix specifies that the α -carbon atom has the configuration of the *l* family and the β -carbon atom that of the *d* family. However, the name constituted an exception to the otherwise invariable rule in amino acid nomenclature regarding the relation of the prefix to the configuration of the α -carbon atom. Confusion has already arisen since papers have appeared in which the isomer of threonine derived from proteins has been erroneously designated as *l*-threonine or *l*(-)-threonine.

Comment on the Rules

The present changes in the nomenclature of the amino acids are the outgrowth of discussions on the part of the Editorial Board of The Journal of Biological Chemistry, and the Editorial Committee of the American Society of Biological Chemists, with generous assistance from the Editorial Board of the Biochemical Journal and from the Committee on Nomenclature, Spelling and Pronunciation of the American Chemical Society.

Rule 1 advocates the general use of a prefix to denote the configurational family of the α -carbon atom in the interests of absolute clarity. However, the prefix may be omitted where the context is such that there is no possibility of misunderstanding which isomer is under discussion as, for example, in papers that deal exclusively with the isomers derived from proteins. Similarly, papers that deal with the synthesis or properties of optically inactive mixtures or racemic compounds may likewise make use of the names without the prefix. Nevertheless the practice of employing the prefix at least once, as for example in the introduction or in the summary of the paper, is to be commended. In effect, this rule merely recognizes current custom in biochemical literature.

The plus and minus signs employed in the Wohl and Freudenberg system of nomenclature were chiefly designed to emphasize the fact that the prefix referred only to the configurational family. They showed that a nomenclature different from that previously employed was being used. With the substitution of capital letters, in analogy with the usage in the carbohydrate field, the special meaning of the symbols is sufficiently emphasized and no signs for the rotation are required. Rule 2 thus eliminates these signs together with the need for a convention regarding the solvent to be employed for the observation of the rotation. Where an author wishes to emphasize the actual direction of the rotation, the specific rotation should be given in the text in the conventional symbols with a record of the temperature, the wave-length of the light, the solvent and the concentration of the solution or its density. It should perhaps be pointed out that the present rules have to do only with the *naming* of amino acids. They do not touch upon the techniques for measuring the rotation.

Although the use of *small* capital letters is desirable in printed material mainly for esthetic reasons, typewritten material will of necessity employ ordinary capital letters. In a manuscript prepared for the printer, a double underline indicates that the symbol is to be set with a small capital letter.

The application of Rule 2 in the case of threonine involves a change in the name originally given to the isomer of this substance isolated from proteins. The new name, L-threonine, has been accepted by Professor W. C. Rose, its discoverer.

The fundamental difficulty in the naming of this substance arises from the fact that carbohydrate nomenclature is based upon the glyceric aldehydes, the dextrorotatory isomer being by convention designated D-glyceric aldehyde. The configuration of the *highest numbered* asymmetric carbon atom determines the prefix that is used. If this configuration is that of D-glyceric aldehyde, the carbohydrate is a D-sugar. On the other hand, in the amino acid field, it is the configuration of the *lowest numbered* asymmetric carbon atom, i.e. the α -carbon atom, that determines the prefix. The two systems are thus irreconcilable unless some device such as an additional

symbol is introduced to show that the capital letter prefix in the name L-threonine is used in the amino acid sense rather than in the carbohydrate sense, as might be inferred from the name threonine with its obvious reference to the sugar threose.

Rule 3 is formulated to deal with this situation and should be applied where *any possibility exists of confusion between the meaning of the symbols in the two systems of nomenclature*. The subscripts s and g, respectively for serine and for glyceric aldehyde, were suggested as being suitable by Dr. C. S. Hudson who pointed out that serine is the simplest amino acid that can be regarded as being related to the carbohydrates. The prefix L_s -, pronounced *ell sub ess*, attached to the name of an amino acid as, for example, L_s -threonine, shows unequivocally that the prefix refers to the amino acid system of nomenclature and that it denotes the configuration of the α -carbon atom. Even if carbohydrates are mentioned by name in the context with prefixed capital letters for their configuration, there would be little chance that confusion as to the exact meaning of the symbols might occur. To eliminate even this small chance, the subscript g could be employed with the prefixes for the carbohydrate names.

Custom dictates that the acids derived from the amino sugars shall be named in accordance with carbohydrate nomenclature even though they may be α -amino acids. Accordingly, *in a context that includes the names of amino acids*, glucosaminic acid should be named D_g -glucosaminic acid, the prefix being pronounced *dee sub gee*, in order to make the meaning of the symbols unequivocal. In this substance, the configuration of the α -carbon atom also chances to be that of the D_s family, but in the acid derived from D_g -mannosamine (2-amino-2-desoxy- D_g -mannose), the α -carbon atom has the configuration of the L_s family. Nevertheless, the name D_g -mannosaminic acid (2-amino-2-desoxy- D_g -mannonic acid) should be employed.

The use of the subscript s added to the capital letter prefix in the nomenclature of the amino acids should rarely be necessary in practice even in the case of threonine. However, it must be remembered that the name L-threonine is at best a compromise, in this name, the two systems under which the capital letter is used to denote configuration come into unavoidable conflict. Authors of papers in which any question arises concerning the configuration of the carbon atoms of threonine must therefore use extreme care in order to avoid the possibility of being misunderstood.

Rule 4 provides for the case of amino acids or their derivatives of whatever origin, the configuration of which has not been established. With such substances, the direction of the optical rotation serves to specify the isomer under consideration. Names assigned under this rule would, of course, be superseded as soon as the configuration had been established.

Rule 5 continues present practice in the naming of those few substances

in which there are two asymmetric carbon atoms but which, being symmetrical in structure, are optically inactive by internal compensation. *meso*-Cystine and *meso*-lanthionine are the two best known examples but other cases such as diaminosuccinic acid and djenkolic acid exist. The abbreviation *ms*- may be employed if desired but not the abbreviation *m*- since this is reserved for *mela*.

Rule 6 deals with the amino acids that have two centers of asymmetry in the carbon chain because a substituent group in addition to the α -amino group is present. Four optically active isomers and two optically inactive mixtures or racemic compounds exist. The rule follows the custom, introduced in the case of the isomers of isoleucine, of designating the diastereoisomer of the substance that is obtained from proteins with the prefix *allo*-, and of using the capital letter prefix to denote the configuration of the α -carbon atom. No attempt is made under the present rules to designate the configuration of the second asymmetric carbon atom, since, where this is of importance, the matter can be more simply dealt with in the text.

Rule 7 extends the use of the capital letter system to derivatives, salts and peptides of the optically active amino acids. The configurational designation is placed immediately before the name of the parent amino acid so that no confusion can arise when complex derivatives are named. Rule 7 likewise applies to systematic names.

If strictly interpreted, this rule would require that the isomer of phenylalanine derived from proteins be named β -phenyl-L-alanine since the substance can be regarded as a substitution product of L-alanine. Unfortunately, β -alanine, in which the amino group occupies the β -position, is a well known substance, being found in the form of a radical in certain natural products and also as a product of the decarboxylation of aspartic acid. The use of the name β -phenyl-L-alanine might therefore lead to misunderstanding; it seems wiser to treat the name phenylalanine, given by Schulze in 1881, as an exception to the rule, and regard it as the parent amino acid in naming derivatives.

There are several other cases in which amino acids of importance in protein chemistry have received analogous names which are partly structural, partly trivial; hydroxyproline, hydroxyglutamic acid, hydroxylysine, dihydroxyphenylalanine and diiodotyrosine are the most important. The rules should be applied strictly in these and similar cases, for example, diiodo-L-tyrosine or, more specifically, 3,5-diiodo-L-tyrosine, 4-hydroxy-L-proline, β -3,4-dihydroxyphenyl-L-alanine. Where the exact position of the second substituent group is still unknown or where its position is unimportant to the context, the prefix which shows this position is omitted, for example, hydroxy-L-lysine, hydroxy-L-proline, diiodo-DL-tyrosine, dihydroxyphenyl-L-alanine.

Rule 8 extends the system to those few substances that have been given

trivial names and in which two amino acid groupings with different constitutions occur. Cystathionine is an example that has recently been fully

Amino acid	Prefix			Present rules
	Early literature	Wohl-Freudenberg system		
		Water convention	Acid convention	
Alanine	<i>d</i>	<i>l</i> (+)	<i>l</i> (+)	L-alanine
Arginine	<i>d</i>	<i>l</i> (+)	<i>l</i> (+)	L-arginine
Aspartic acid	<i>d</i>	<i>l</i> (+)	<i>l</i> (+)	L-aspartic acid
Cystine	<i>l</i>	<i>l</i> (-)	<i>l</i> (-)	L-cystine
Cysteine*	<i>l</i>	<i>l</i> (-)	<i>l</i> (+)	L-cysteine
Glutamic acid	<i>d</i>	<i>l</i> (+)	<i>l</i> (+)	L-glutamic acid
Histidine*	<i>l</i>	<i>l</i> (-)	<i>l</i> (+)	L-histidine
Hydroxyproline ¹	<i>l</i>	<i>l</i> (-)	<i>l</i> (-)	hydroxy-L-proline
Isoleucine ²	<i>d</i>	<i>l</i> (+)	<i>l</i> (+)	L-isoleucine
Leucine*	<i>l</i>	<i>l</i> (-)	<i>l</i> (+)	L-leucine
Lysine	<i>d</i>	<i>l</i> (+)	<i>l</i> (+)	L-lysine
Methionine*	<i>l</i>	<i>l</i> (-)	<i>l</i> (+)	L-methionine
Ornithine ³	<i>d</i>	<i>l</i> (+)	<i>l</i> (+)	L-ornithine
Phenylalanine	<i>l</i>	<i>l</i> (-)	<i>l</i> (-)	L-phenylalanine
Proline	<i>l</i>	<i>l</i> (-)	<i>l</i> (-)	L-proline
Serine*	<i>l</i>	<i>l</i> (-)	<i>l</i> (+)	L-serine
Threonine ⁴	<i>d</i>	<i>d</i> (-)	<i>d</i> (-)	L-threonine
Thyroxine ⁵	<i>l</i>		<i>l</i> (-)	L-thyroxine
Tryptophan*	<i>l</i>	<i>l</i> (-)	<i>l</i> (+)	L-tryptophan
Tyrosine	<i>l</i>	<i>l</i> (-)	<i>l</i> (-)	L-tyrosine
Valine	<i>d</i>	<i>l</i> (+)	<i>l</i> (+)	L-valine

* The amino acids marked with an asterisk are levorotatory in neutral aqueous solution and dextrorotatory in acid aqueous solution.

¹ The configuration of the γ -carbon atom of hydroxy-L-proline has been shown by Neuberger (J. Chem. Soc., 1945, 429) to be that of the *D* family.

² The configuration of the β -carbon atom of isoleucine has not been established with certainty.

³ Ornithine has not been isolated from proteins save after hydrolysis with alkali. Under these circumstances, DL-ornithine is obtained by secondary decomposition and racemization from L-arginine. L-Ornithine has, however, been isolated from tyrocidine (Gordon, Martin and Syngé, Biochem. J., **37**, 313 (1943)) and from gramicidin-S (Syngé, Biochem. J., **39**, 363 (1945)).

⁴ The configuration of the β -carbon atom of threonine is that of the *D* family.

⁵ Thyroxine is too insoluble in water or in acid for convenient observation of the rotation; it is levorotatory in alkaline solution.

worked out. In this substance, a cysteine and a homocysteine radical are combined through a single sulfur atom. Octopine is a case in which the isomers still remain to be described. If the convention is adopted that the

configurational designation shall be that of the smaller of the two amino acid radicals and, in addition, the prefix allo- (this prefix is to be preferred to iso-) is employed to denote the diastereoisomeric analogue of the compound to which the trivial name was first assigned, an unequivocal set of designations becomes possible. The systematic name must, however, be relied upon if the configurations of *both* asymmetric carbon atoms are to be specified.

The accompanying table shows the names of the isomers of the more important amino acids derived from proteins under the systems of nomenclature previously in use and under the present rules.

HUBERT BRADFORD VICKERY

*For the Editorial Board of The
Journal of Biological Chemistry*

METHIONINE FORMATION BY TRANSMETHYLATION IN VITRO

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We reported in a previous communication (1) that guanidoacetic acid is methylated to creatine by rat liver slices in the presence of choline and either homocysteine or homocystine. Choline, homocystine, or homocysteine alone had little or no effect. These observations closed the gap which had existed between the evidence from tissue slice experiments on the one hand (2) and that from experiments *in vivo* on the other (3-5) regarding the transfer of choline methyl in the formation of creatine.

Two transmethylation reactions, one in which methionine, the other in which creatine is formed, were thus exposed for study. We had observed that the rate of methylation of guanidoacetic acid by choline is as fast or faster with homocystine than with homocysteine as the methyl carrier (1). This was surprising, as it might have been expected, *a priori*, that homocysteine would be the more effective methyl carrier, and it suggested that the two transmethylation reactions may have different characteristics. We had found an indication earlier (2) that the methylation of guanidoacetic acid by methionine is not a simple methyl transfer between the two substances in that the reaction is inhibited when respiration is inhibited, whether by anaerobiosis or by oxidation inhibitors. We undertook a study of methionine formation by transmethylation in rat liver and kidney slices and homogenates to obtain more information on this question, and also because of the physiological significance of the reaction in itself, to which no *in vitro* studies had as yet been devoted.

Methods

The animals used were adult white rats which had been bred from the Wistar Institute strain. They were killed by stunning; the liver and kidney tissue were sliced free-hand with a straight edge razor to an average thickness of 0.4 mm. The slices were rinsed in Krebs and Henseleit (6) Ringer's solution without the bicarbonate and finally suspended in the same Ringer's solution with such a concentration of bicarbonate that the pH, when equilibrated at 38° with 5 per cent carbon dioxide, was 7.4. The reactants were dissolved in the latter version of the Krebs-Henseleit bicarbonate Ringer's solution.

The reaction vessels were 20 ml. beakers. They were placed in a closed chamber which was partially immersed in a water bath. The chamber was provided with a gas inlet tube through which the gas mixture was passed under slight pressure; the excess gas escaped under water through a slit in the side. The chamber could hold thirty beakers. When the latter were in place, the gas mixture was blown through vigorously for 10 minutes, after which it was slowed down. Through the period of gassing and afterwards the chamber containing the beakers was rocked at a rate of about 80 cycles per minute. By prior tests it was ascertained that the evaporation from the reaction vessels in a 4 hour run was negligible. The temperature of the water bath was 38°.

When homogenates were used, the tissue was removed and, without chilling, cut into small pieces and homogenized in the apparatus of Potter and Elvehjem (7) in isotonic phosphate-Ringer's solution (6) at pH 7.4. The homogenate was strained through two layers of cheesecloth.

The lyophilized enzyme was prepared as follows. The tissue was sliced, the slices rinsed as described above, and then frozen on a block of solid carbon dioxide, transferred to flasks previously cooled to the temperature of solid carbon dioxide, and then dried under a high vacuum with the water trap kept in a bath of solid carbon dioxide in methyl cellosolve (Mefford). The thoroughly dried tissue was pulverized.

The first experiments were carried out with preparations of DL-homocysteine and of DL-homocystine, generously given us by Professor V. du Vigneaud.

Later it seemed desirable to use the natural isomers. The starting material for their preparation was L-methionine obtained from casein by the method of Hill and Robson (8) with the following modifications. After the usual acid hydrolysis, removal of excess HCl, adjustment of the pH to 2.4, decoloration with charcoal and separation of the tyrosine, the pH was adjusted to 6.0 and the iron (presumably in the HCl and NaOH used) was removed by treatment with H_2S . The removal of the iron clarified the solution further and facilitated the crystallization of the leucine and methionine. Methionine was separated from the leucine by the mercury precipitation procedure described by Hill and Robson; after removal of the mercury the solution of impure methionine hydrochloride was concentrated nearly to dryness, dissolved in alcohol, and the solution brought to pH 6.0 with concentrated NaOH instead of with pyridine, as recommended by Hill and Robson, and set away overnight in the ice box. Methionine which had crystallized out was removed, the mother liquor concentrated, alcohol added to 80 per cent concentration, the pH adjusted to 6.0 with concentrated NaOH, and set away in the ice box overnight. The second crop of methionine thus obtained was added to the first. The methionine

was purified further by a second mercury precipitation as described by Hill and Robson, and recovered from the mercury precipitate by the same procedure as after the first mercury precipitation. The methionine was purified finally by two precipitations with alcohol from water.

L-Homocystine was prepared from L-methionine by the method of Butz and du Vigneaud (9), as modified by du Vigneaud and Patterson (10).

L-Homocysteine was prepared from L-homocystine by reduction with sodium in liquid ammonia, following the procedure of Riegel and du Vigneaud (11).

Choline hydrochloride and betaine hydrochloride were used from several commercial sources. Dr. N. Horowitz kindly supplied specimens of the following compounds used: di- and monomethylethanolamine, monoethylmethyl- and diethylmonomethylhydroxyethylammonium chloride, arsenocholine, phosphorylcholine, and homocysteinethiolactone.

Methionine was determined by an adaptation of the method of McCarthy and Sullivan (12) which made the method more sensitive but somewhat less specific. The reduction in specificity was not significant under our experimental conditions, *i.e.* Ringer's solution in which tissue slices or liver homogenates had been incubated and in which the methionine did not exceed 10 mg. per cent and the total of other amino acids 10 mg. per cent. The procedure was as follows: to 4 ml. of Ringer's solution containing the slices were added 0.4 ml. of 30 per cent trichloroacetic acid; 2 ml. of filtrate were transferred to a 20 ml. beaker; 0.2 ml. of 5 N NaOH followed by 0.1 ml. of 1 per cent freshly made sodium nitroprusside were then added. The resulting solution was warmed with gentle rocking in a water bath at 38° for 8 minutes, after which it was cooled in ice water until the temperature was below 4°, and then 1 ml. of an acid mixture consisting of 9 volumes of concentrated HCl and 1 volume of 85 per cent H₃PO₄ was added with shaking. The beaker was again placed in ice water for 1 to 2 minutes, after which it was warmed to room temperature and then transferred to a vacuum desiccator in which the CO₂ was removed by 3 minutes suction. The color was then measured at 510 m μ in a Beckman spectrophotometer. Methionine standards were run simultaneously with the unknowns; the standard solution containing no methionine was used as the blank in the spectrophotometer. A straight line relation is obtained between the logarithm of the transmission and the concentration of methionine over the range, 0 to 10 mg. per cent.

The method was tested by incubating known amounts of added methionine in Ringer's solution with liver slices for 4 hours at 38°. The assay gave 98 per cent of the added methionine over a range of 0 to 10 mg. per cent after deducting the methionine liberated by the slices. The deficiency of 2 per cent was probably the result of oxidation of the added methionine.

The above modification of the McCarthy-Sullivan method for the determination of methionine may be used only when the total concentration of non-methionine amino acids is of the order of 0.001 M or less. When the latter are present in higher concentration, the original recipe of McCarthy and Sullivan is advisable.

Results¹

In Table I are summarized the results of experiments which show the formation of methionine by rat liver slices from homocysteine or homocystine and a suitable methyl donor. Larger yields of methionine were obtained under low oxygen tension (4 per cent O₂), or anaerobically, than under 95 per cent O₂, from homocysteine than from homocystine, whether under aerobic or anaerobic conditions, and with betaine than with choline as the methyl donor. The superiority of betaine over choline was greatest under anaerobic conditions and least under 95 per cent oxygen. The effect of oxygen tension on the yield of methionine was far less when choline was the methyl donor than when betaine was used.

The following facts offer a qualitative and at least partial explanation of the higher yields of methionine under nitrogen than under oxygen and of the greater relative effect of anaerobiosis with betaine than with choline as the methyl donor. Homocysteine is more stable under nitrogen than under oxygen, presumably because it is protected from oxidation.² Choline is rapidly oxidized by rat liver slices and homogenates to betaine on the alkaline (pH 7.8) and to betaine aldehyde on the acid (pH 6.7) sides of neutrality (13, 14). In its oxidation to betaine choline becomes a more effective methyl donor for methionine formation. Aerobiosis and anaerobiosis thus have opposite effects on the methyl donor and methyl acceptor when choline is the methyl donor; hence the small difference in the yield of methionine under the two conditions. Betaine is not oxidized by rat liver

¹ Nearly all the results reported here were first obtained with the DL forms of homocysteine and of homocystine. The experiments were repeated with the natural isomers, and, as there were no essential differences between the two sets of experiments, only those of the latter are reported.

² That homocysteine is protected by anaerobiosis was shown by the difference in the intensity of the transitory purple color after the addition of alkali and nitroprusside in the first stage of the methionine determination. In the experiments under 95 per cent oxygen with homocysteine the purple color was faint, and deep when they were conducted under nitrogen, even though more homocysteine was converted to methionine in the latter solutions. Similar results were found with homocystine; no purple color was seen after experiments under 95 per cent oxygen, a faint purple after the anaerobic experiments. In the latter case the color was less intense than when homocysteine was used under the same conditions. The purple color given by homocystine in the anaerobic experiments was qualitative evidence that it was reduced to homocysteine.

slices or homogenates (13, 15). The greater yields of methionine under anaerobic conditions with betaine are then to be ascribed to the greater stability of the methyl acceptor. This explanation is offered only pro-

TABLE I

Methylation of L-Homocysteine and of L-Homocystine by Choline and by Betaine

Rat liver slices; 4 hours, 37.5°. The results are expressed as micrograms of methionine per 100 mg. of tissue (dry weight).

Composition of gas mixture	95 per cent O ₂ + 5 per cent CO ₂			91 per cent N ₂ + 4 per cent O ₂ + 5 per cent CO ₂		95 per cent N ₂ + 5 per cent CO ₂	
	28.8	19.1	9.6	19.1	9.6	19.1	9.6
Initial concentration of methyl donor, mg. %							
Tissue alone	75	75	75	92	55	72	119
" + homocysteine	104	104	104	259	207	183	223
" + " and choline	207	167	147	362	338	279	271
Tissue + homocysteine and betaine	254	215	163	716	470	1019	721
Tissue + homocystine	75		75	194	107	92	167
" + " and choline	107		107	239	211	139	215
Tissue + homocystine and betaine	115		115	279	235	633	525
Tissue + choline	75	75	75	95	55	72	119
" + betaine	75	75	75	92	55	68	124

Each of the figures in the table is the average of a triplicate. L-Homocysteine and L-homocystine were used. The initial concentration of each was in every case 12.5 mg. per cent, and the volume of the reaction mixture was 4 ml. In any one experimental run the gas mixture and the concentration of the methyl donor were the same; the slices were obtained from the liver of one animal or from the pooled slices of the livers of two animals. The dry tissue weight in each reaction vessel was within the range, 15 to 30 mg. A number of experiments were carried out to observe the effect of varying the oxygen tension in the gas mixture, with slices from one animal. The results were essentially the same as those shown above; they were not selected for inclusion in Table I because, for physical reasons, the number of controls were limited, and the homocysteine and homocystine experiments had to be run separately in the latter experiment.

visionally; it needs the support of data on the rates of all the reactions and side reactions involved.

In most, but not all, experiments there was an increase in methionine over the tissue blank when only homocysteine or homocystine was added, i.e. not accompanied by an additional methyl donor. Presumably the methylation was effected by methyl donors in the liver slices; these were not removed even by washing the slices in Ringer's solution for $\frac{1}{2}$ hour.

In our studies on the methylation of guanidoacetic acid (1, 2) we found a considerable methylation by liver slices, even when no methionine was added. Until a sufficiently sensitive method for the determination of methionine was available, we were uncertain whether this "tissue" methylation might be ascribed to methionine liberated by the liver slices during the period of incubation or whether it was necessary to invoke some other methyl donor. The method for the determination of methionine used in this study was sufficiently sensitive. With it we have found that rat liver slices liberated methionine, under the conditions of the experiments on the methylation of guanidoacetic acid, in concentrations which ranged from 0.2 to 1.0 mg. per cent; in 4 hours the total free methionine increased usually to about 3 times the zero time value. As the "tissue" methylation of guanidoacetic acid could be accounted for by a free methionine concentration of 3×10^{-6} M, *i.e.* approximately 0.5 mg. per cent, the methionine liberated by rat liver slices is sufficient, therefore, to account for the rates of methylation of guanidoacetic acid observed when no methionine is added to the Ringer's solution.

Cyanide, azide, arsenite, and arsenate do not inhibit the methylation of homocysteine or of homocystine (Table II). In fact arsenite increased the yield of methionine even under 4 per cent oxygen. The non-susceptibility of this transmethylation to oxidation inhibitors is in accord with its independence of oxygen.

The possibility suggested itself, in view of the superiority of betaine to choline as a methyl donor in methionine formation, that the methylating action of choline may be via betaine; *i.e.*, that betaine is the immediate methyl donor to homocysteine or homocystine and that the methyl group lost by such a donation is replenished by choline. The possibility was tested in a number of experiments in which varying small amounts of betaine were added to the reaction mixture containing the methyl acceptor and choline. The choline concentration in these experiments was 3 times the molecular equivalent of the homocysteine and 6 times that of homocystine; the betaine concentration was varied from one-fifth to one-hundredth that of the choline. The results obtained argue against a carrier function of betaine. The effect of choline plus betaine was always less than the sum of their separate effects.

In addition to betaine and choline a number of other possible methyl donors were tested. As Table III shows at the end of the 4 hour incubation with liver slices at 38°, no more and in some cases less methionine was found than when the methyl acceptor alone was added to the Ringer's solution. The other possible methyl donors tested were dimethylethanolamine, monomethylethanolamine, diethylmethylhydroxyethylammonium chloride, monoethyldimethylhydroxyethylammonium chloride, arsenocholine, and phosphorylcholine.

These results illustrate one of the limitations of the tissue slice technique. The tissue slices contained significant amounts of methyl donors, even after being washed $\frac{1}{2}$ hour in large amounts of Ringer's solution. The failure of the five possible methyl donors other than betaine or choline to add, under our experimental conditions, to the rate of methylation effected by the methyl donors in the slices does not, in itself, indicate that they could not

TABLE II

Effect of Oxidation Inhibitors on Methylation of L-Homocysteine and of L-Homocysteine by Choline and by Betaine

Rat liver slices; 4 hours, 37.5°. The results are expressed as per cent of the methionine found after addition of homocysteine plus choline in the absence of an oxidation inhibitor.

Composition of gas mixture	95 per cent O ₂ + 5 per cent CO ₂					91 per cent N ₂ + 4 per cent O ₂ + 5 per cent CO ₂			
	None	Cyanide	Azide	Arsenite	Arsenate	None	Azide	Arsenite	Arsenate
Tissue alone	46	50	44	28	35	21	15	4.5	19
" + homocysteine and choline	100	116	99	163	100	100	75	145	114
Tissue + homocysteine and betaine	115	121	103	324	119	112	87	155	121
Tissue + homocysteine and choline	61	67	63	78	63	59	57	70	52
Tissue + homocysteine and betaine	64	74	66	113	75	65	60	81	61

L-Homocysteine and L-homocysteine were used at a concentration of 12.5 mg. per cent. The volume of the reaction mixture was 4 ml. In the experiments with 95 per cent oxygen the concentration of methyl donor was 28.8 mg. per cent and with 4 per cent oxygen, 9.6 mg. per cent. The oxidation inhibitors were used in the form of their sodium salts and at a concentration of 0.001 M. In every experiment the controls consisted of tissue alone, tissue plus homocysteine, and tissue plus homocysteine; the controls and the different reaction mixtures, all in triplicate, were run simultaneously in the absence and in the presence of one of the above oxidation inhibitors.

do so *in vivo*. One possibility is that under our experimental conditions the above substances are not transformed to betaine or to choline fast enough to make their contribution to that of the methyl donors in the slices noticeable.

The results summarized in Table III are in accord with those on growth (4, 16) in that none of the compounds which were found to be incapable of supporting growth with homocysteine exerted any additive methylating effect under our experimental conditions. Monoethyldimethylhydroxyethylammonium chloride and phosphorylcholine will support growth with homocysteine presumably because they are converted to choline (or

betaine) *in vivo*; they exerted no significant additive methylating effects in our experiments because, presumably, they were not converted to betaine or to choline sufficiently rapidly to augment noticeably in short period experiments the effect of the methyl donors in the slices.

TABLE III

Relative Rates of Methylation of L-Homocysteine, L-Homocystine, and of Homocysteine thiolactone by Various Possible Methyl Donors

Rat liver slices, 4 hours, 37.5° Excess methionine over tissue blank with homocysteine alone taken as 100

Composition of gas mixture	95 per cent O ₂ + 5 per cent CO ₂	91 per cent N ₂ + 4 per cent O ₂ + 5 per cent CO ₂	95 per cent N ₂ + 5 per cent CO ₂
Homocysteine	100	100	100
“ + choline	217	220	270
“ + betaine	347	633	1905
“ + dimethylethanolamine	101	95	90
“ + monomethylethanolamine	65	99	93
“ + diethylmonomethylhydroxyethylammonium chloride	119	91	-20
Homocysteine + monoethyldimethylhydroxyethylammonium chloride	150	100	-120
Homocysteine + arsenocholine		76	
“ + phosphorylcholine		94	
Homocystine	55	51	60
“ + choline	123	102	104
“ + betaine	165	180	208
“ + dimethylethanolamine	-42		27
“ + monomethylethanolamine	-138		-240
“ + diethylmonomethylhydroxyethylammonium chloride	-138		0
Homocystine + monoethyldimethylhydroxyethylammonium chloride	54		-20
Homocystine + arsenocholine		21	
“ + phosphorylcholine		11	
Homocysteine thiolactone		72	
“ + choline		92	
“ + betaine		254	
“ + arsenocholine		20	
“ + phosphorylcholine		49	

Table III also contains a summary of results obtained with homocysteine-thiolactone. It was transformed to methionine at a rate less than that of homocysteine (50 to 75 per cent) and slightly superior to that of homocystine. Betaine was in this instance also a more effective methyl donor than choline.

Homocysteinethiolactone can replace homocystine for rat growth (17). *Methionineless* mutants of *Neurospora* which can utilize homocystine as a substitute for methionine use homocysteinethiolactone better, and homocysteine still better.³ Our findings with homocysteinethiolactone and these are in accord.

Homogenized or lyophilized and dialyzed rat liver retain the ability to catalyze the methylation of homocysteine and of homocystine. Some results with lyophilized liver are given in Table IV. Some activity is lost

TABLE IV

Methylation of L-Homocysteine and of L-Homocystine with and without Choline and Betaine by Lyophilized Rat Liver

Nitrogen, 4 hours, 38°. The figures are mg. per cent of methionine in the trichloroacetic acid filtrates.

Reaction mixture	Undialyzed powder		Dialyzed enzyme used in each test equivalent to 100 mg. powder			
	25 mg	100 mg	Alone	Plus dialysate	Plus ash	Plus dialysate plus ash
Blank	0.6	2.5	2.7	3.1	2.7	3.2
Homocysteine	1.25	6.0	3.5	4.1	3.2	4.1
“ + choline	1.40	6.6	3.6	4.3	3.0	4.2
“ + betaine	3.00	9.8	6.4	5.5	5.7	6.1
Homocystine	0.6	4.2				
“ + choline	0.7	4.8				
“ + betaine	0.8	7.7				
Choline	0.55	2.6	2.7	3.0	2.8	3.2
Betaine	0.60	3.0	2.7	3.2	3.1	3.2

The powder and reactants were dissolved in 0.1 M phosphate buffer at pH 7.0. The final volume was 4 ml. Concentrations of homocysteine and of homocystine were 12.5 mg. per cent, of choline and betaine 37.5 mg. per cent.

in lyophilization and a further loss occurs in dialysis. The loss in activity indicates that there may be two mechanisms, one which uses choline, the other betaine; the former appears to suffer more loss in dialysis than the latter.

We have carried out a number of experiments with slices of rat kidney cortex. Methyl donors within (*i.e.* not washed out of) the kidney slices effected about the same amount of methylation of homocysteine and homocystine as did liver slices. Choline and betaine exerted only slight additive methylating effects. Unlike liver, kidney slices were more effective under aerobic than anaerobic conditions.

³ Horowitz, N., unpublished experiments, private communication.

DISCUSSION

The results of experiments reported here have established that there are at least two categories of transmethylation reactions: One, of which the methylation of guanidoacetic acid to creatine is an example, depends on oxygen and is inhibited by oxidation inhibitors; the other, represented by the formation of methionine from homocysteine or homocystine as the methyl acceptor and choline or betaine as the methyl donor, does not require oxygen and is not inhibited by oxidation inhibitors. In the first category methionine can act as a methyl donor but choline and betaine cannot unless homocysteine or homocystine is present to serve as a methyl carrier. Still another difference is that liver homogenates are unable, with or without added methionine, to methylate guanidoacetic acid; they retain their ability to catalyze methionine formation by transmethylation.⁴

In the first category the direction of the methyl transfer is from an S-methyl to an N-methyl and the reverse in the second category. This distinction may not be fundamental; it seems advisable to suspend judgment on this point at present.

Perlzweig *et al.* (18) reported that rat liver slices can methylate nicotinamide. The reaction is strictly aerobic, requires unbroken cells, is usually but not always accelerated by the addition of methionine, and the direction of the methyl transfer is from S-methyl to N-methyl, characteristics which place this methylation in the same category as the methylation of guanidoacetic acid. The two methylations are similar also in that they are restricted to the liver.

Stetten (19) proposed a scheme of choline formation from betaine in which betaine is demethylated to glycine, the latter is reduced to ethanolamine, which is then methylated to choline by methionine. The direct participation of methionine was not established and the pathway of betaine methyl was left open. The observations reported here show that a methyl group of betaine is available for choline formation by way of its incorporation into methionine and thus account for the physiological properties of betaine associated with its possessing a labile methyl group. Other possibilities are not excluded.

Chandler and du Vigneaud (20) found that there was a lag of several days before growth was resumed after betaine was added to a diet containing homocystine and devoid of or very low in methionine and choline, whereas growth was resumed immediately after the addition of choline. Even after the lag, betaine was somewhat less effective than equivalent amounts of choline. Griffith and Mulford (21) found that 3 to 4 mg. of methionine

⁴ After this manuscript was submitted for publication we found that the methylation of guanidoacetic acid by methionine occurs in guinea pig liver homogenates if adenosine triphosphate is provided.

or of betaine were required to give the same effect as 1 mg. of choline in preventing fatty liver or hemorrhagic kidney. As an explanation of the lesser efficiency of betaine than choline, the evidence Stetten obtained with N^{15} excluded direct reduction of betaine to choline. Our observations indicate that one route by which betaine methyl is made available for growth and for lipotropic purposes is via its incorporation into methionine, and hence its lower efficiency than choline for the latter function. None of the above observations excludes the possibility that ethanolamine may be methylated by betaine directly.

When choline is oxidized to betaine, at least one of its methyl groups remains labile via incorporation into methionine. There is considerable evidence that choline can be oxidized to betaine in the body. Rat liver slices and homogenates rapidly oxidize choline to betaine aldehyde and betaine (13, 14); betaine has been found in considerable amounts in animal tissues (22-25) and has been isolated from the urine of animals on a betaine-free diet after being fed large amounts of choline (26).

Although methyl transfer proceeds more rapidly from betaine than from choline, it seems unlikely from the evidence at hand that oxidation of choline to betaine is obligatory for this purpose. If it were, there should be little or no methionine formation with choline as the methyl donor under anaerobic conditions. Choline is effective under these conditions.

SUMMARY

1. Rat liver slices methylate homocysteine, homocystine, and homocysteinethiolactone to methionine. Choline or betaine can serve as the methyl donor.

2. The speed of the reaction with each of the three methyl acceptors is fastest with homocysteine and slowest with homocystine. It is faster with betaine than with choline as the methyl donor.

3. A number of possible methyl donors were tested; other than betaine and choline, none exerted a methylating effect which was additive to that of the methyl donors retained in the slices.

4. These transmethylation reactions are independent of oxygen and are not inhibited by oxidation inhibitors.

5. Catalytic activity is retained in homogenized and in lyophilized rat liver.

6. There are two (at least) categories of methyl transfer reactions. One is dependent on oxygen, is inhibited by oxidation inhibitors, and catalytic activity is lost by homogenization and is not restored by adding methionine, which accelerates the reaction with slices. In this category is the methylation by methionine of guanidoacetic acid to creatine and of nicotinamide to N^1 -methylnicotinamide. Characteristics of the second category are

independence of oxygen, nonsusceptibility to oxidation inhibitors, and persistence of catalytic activity after cell structure is destroyed. In this category is methionine formation by the methylation of homocysteine, homocysteinethiolactone, or homocysteine by betaine or choline.

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THE METHIONINE AND CYSTINE CONTENT OF HEN'S EGGS*

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At the 1941 annual meeting of the American Society of Biological Chemists, one of us presented evidence that the amino acid composition of the egg may be influenced by feeding the hen a diet rich in protein (1). We now offer experimental results showing how the methionine and cystine content of the egg can be changed by varying the protein in the hen's diet according to its high or low methionine content.

McFarlane, Fulmer, and Jukes (2) found no changes in the amino acid composition of the egg proteins when they varied the basal diet of the hens from 10 to 16 per cent crude protein. A higher protein supplement as a dietary influence would have been more appropriate to test the constancy of the amino acid composition of the egg proteins.

Calvery and Titus (3) did not observe any change in the amino nitrogen distribution in egg albumin and vitellin prepared from eggs laid by hens fed a diet in which the protein was furnished exclusively by wheat, corn, or soy bean. Similar feeding experiments were carried out with pigeons by Gerber and Carr (4). They observed differences in the N distribution in the egg proteins (Van Slyke) and in the immunological behavior in rats sensitized to egg proteins. Their finding points to the probability of change in the amino acid composition of the egg protein induced by the ingestion of the various feeds.

If the amino acid composition of the different egg proteins is considered constant, then an increase in an amino acid in the egg may mean that the protein content of the egg has increased or that the proportions of the various egg proteins have changed. An increase in total protein content would naturally bring about proportional increases in all of the amino acids, and the cystine-methionine ratio would not be changed. However, we will show that the cystine-methionine ratio does not remain constant and that the levels of these two amino acids are independent of the total N in the egg.

Variations in the protein components of egg white were noted by Longworth, Cannan, and MacInnes (5). They found that the albumin content

* This is the ninth in a series of papers on amino acids in staple foods.

of the egg white varied from 60 to 70 per cent and the conalbumin from 13 to 18 per cent.

It is known that the ratio of white to yolk changes in eggs of an individual hen. The influence of the diet and the laying season on these changes are not known.

Because of the high biological value of egg proteins, the egg is one of the most valuable supplementary foods in human nutrition. A change brought about in the composition of the egg in amino acids, especially the indispensable ones, is a significant finding in relation to its biological value. This may also be important for the developing chick, since the materials stored in the egg are the only available nutrients for the growing embryo.

EXPERIMENTAL AND DISCUSSION

Rhode Island Red hens in their 2nd laying year were used in these experiments. The chickens were kept in laying batteries. They were on a low protein diet for at least a month before they were fed the high protein diet. The low protein diet had the following composition in percentages: ground yellow corn 87, alfalfa leaf meal 5, steamed bone meal 4.5, butyl fermentation solubles (250 γ of riboflavin per gm.) 0.5, limestone flour 2, manganized salt (6 per cent MnSO_4) 0.5, iodized salt 0.2, vitamins A and D oil (400 A. O. A. C. units of vitamin D and 2000 I.U. of vitamin A per gm.) 0.3, corn-starch 6.0. This low protein diet contained approximately 9 gm. of crude protein, 0.18 gm. of cystine, and 0.26 gm. of methionine per 100 gm. of feed. Three high protein diets were prepared from the low protein formula by replacing 20 parts of corn-meal in two of the diets with casein or gelatin, and in the third, 50 parts of corn-meal with soy bean meal. Among the diets used in our experiments, the casein-high protein diet was the richest in methionine (0.75 per cent), but rather low in cystine (0.22 per cent). The soy bean-high protein diet contained equal amounts of methionine and cystine (0.4 per cent), and the gelatin-high protein diet had 0.36 per cent of methionine, but less cystine (0.16 per cent) than that present in the low protein feed.

The eggs were collected and weighed daily, and if not hydrolyzed on the same day, they were kept at 5° in a cold storage room until analyzed. When additional methionine was fed, the quantities stated in Figs. 1 and 2 were mixed in approximately 30 gm. of the high protein experimental diet and were fed to the chickens. When this was consumed, the chickens were allowed to feed *ad libitum* on the experimental diet for the remainder of the day.

The methods used for the determination of cystine and methionine have been published (6, 7). Therefore, the remarks concerning both methods will be limited to the details pertinent to the analysis of eggs. The eggs

were opened at the small end, and the contents were allowed to flow directly in the hydrolyzing flask (300 cc. round bottom Pyrex). The crushed shell was pushed in the flask and then 50 cc. of concentrated HCl were added. The action of the HCl with the egg shell in the presence of the egg contents caused foaming, but when the flask was shaken gently three or four times during an hour the foaming subsided. (The use of antifoaming agents, such as butyl or caprylic alcohol, is not recommended.) Then the flask was placed in an oil bath and the contents refluxed for 24 hours at the tem-

TABLE I
Relationship between Egg Weight and Nitrogen, Cystine, and Methionine Content

Hen's diet	Egg weight	Egg Content			Hen's diet	Egg Weight	Egg Content		
		Nitro- gen	Cystine	Meth- ionine			Nitro- gen	Cystine	Meth- ionine
	gm.	mg.	mg	mg		gm.	mg.	mg.	mg
Low protein	55 1	1003	144	156	High protein (soy bean)	58	978	166	198
	56 4	1045	138	184		59.3	952	152	180
	61.1	1124	156	200		59.7	978	162	185
High " (gelatin)	47.7	874	145	132		60.3	1050	178	210
	49.5	863	133	140		60 3	1006	154	188
	52 6	940	128	160		60.9	1009	132	158
	55.1	1014	157	163		61.3	1068	167	192
	56 5	975	140	199		61.8	1014	171	184
High protein (casein)	65 6	1310	173	234		62.2	1076	163	203
						62.5	1035	153	200
High protein (soy bean + 0.1 gm. DL- methionine)	53 1	996	146	156		62.5	1099	167	236
	58.1	939	156	186		64.3	1133	181	225
	59.2	1124	171	190					
	60.4	967	156	195					
	60.4	1011	184	204					
	60 5	982	161	190					
	61 3	1039	163	207					
	61 5	1149	151	188					
	63 0	1222	147	207					

perature of boiling hydrochloric acid. After hydrolysis the total volume of the egg hydrolysate (100 to 125 cc. generally) was determined at room temperature. The hydrolysate was then filtered and suitable aliquots were taken for cystine, methionine, and nitrogen determinations.

In the 5 cc. aliquot taken for cystine, it was not necessary to drive off the excess acid by boiling. This 5 cc. aliquot was diluted to a 25 cc. volume and a 10 cc. aliquot of this was used for the cuprous-cysteine separation. The recommended quantity of acetate buffer was ample to take care of the pH requirement (6). It is expedient to use 20 mg. of carboraffin at the final

stage of Cu precipitation with sodium thiosulfate to remove a yellowish pigment usually present in egg hydrolysates

A 10 cc. aliquot of the egg hydrolysate was used for the methionine determination (7). Duplicate samples of 1 cc. of 1:10 egg hydrolysate were taken for nitrogen determination by the micro-Kjeldahl method of Pregl as modified by Clark (8).

The three proteins, casein, soy bean, and gelatin, which we chose to enrich the hen's diet, differed in respect to their origin (animal and plant)

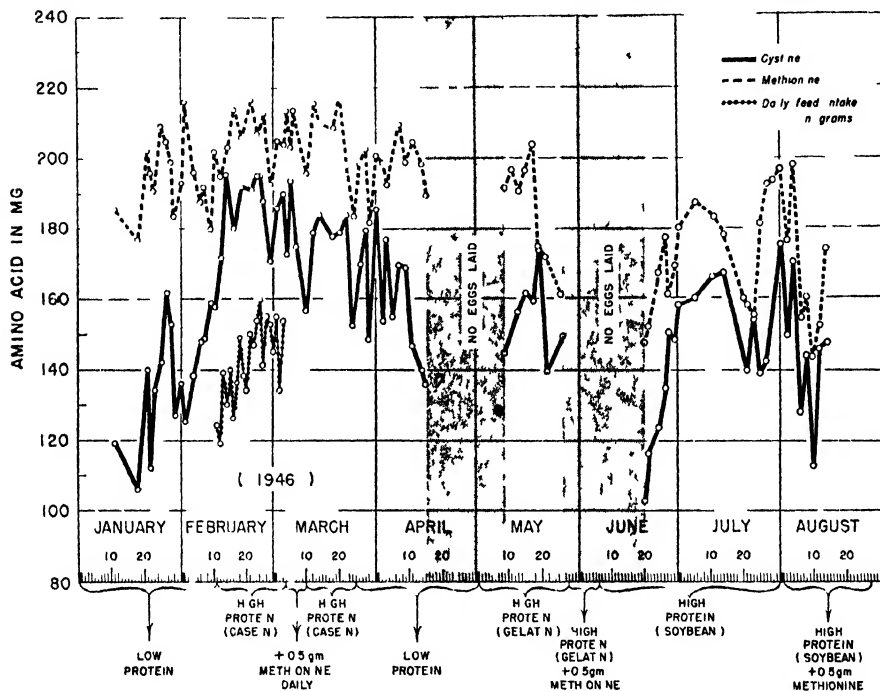


FIG. 1 The influence of dietary protein and methionine supplementation on the cystine and methionine content of the egg (Hen 1)

and also in their methionine and cystine content. There were changes in the weight of the egg and in the cystine, methionine, and nitrogen contents of the eggs laid by a hen which was fed a high and low protein diet. However, there was no definite consistent relation between the weight of the egg and its methionine, cystine or nitrogen content (see Table I).

Although the data arranged according to increasing egg weights show no definite proportionality between the weight of the egg and its nitrogen content, it may be noted that the larger eggs (over 60 gm) contain more nitrogen than do the smaller eggs (under 50 gm).

Fluctuations are present in the methionine and cystine content of the eggs obtained on the low protein as well as on the various high protein diets, as shown in Figs. 1 and 2. Furthermore, an increase in methionine was not always followed by an increase in cystine and vice versa.

We assume that these fluctuations are normal physiological variations that may be caused by the limitation of the necessary components required

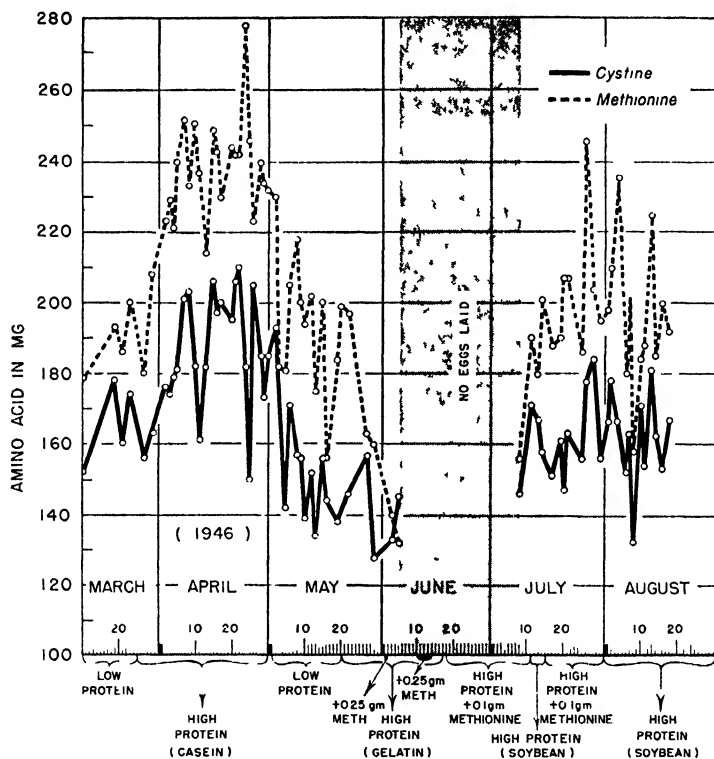


FIG 2 The influence of dietary protein and methionine supplementation on the cystine and methionine content of the egg (Hen 2)

for the formation of the proteins of the yolk and white. This limitation is undoubtedly related to feed intake. The data in Fig. 1 indicate the relationship between the level of methionine and cystine in the eggs and the daily feed intake of a hen.

While theoretically the hen could furnish methionine or cystine from its own reserve protein to complete the synthesis of egg protein, this does not seem to be the case. When the dietary protein is incomplete (20 per cent gelatin in the diet), or when the protein in the diet is low, egg production

stops. Apparently, the hen relies on the constituents of the feed for the synthesis of egg protein.

The data showing the average methionine and cystine values and the weights of the eggs from hens on the low protein and the high protein diets are summarized in Table II.

TABLE II
*Influence of Diet on Weight and Cystine and Methionine Content of Hen's Eggs
(Average Values and Standard Errors)**

Hen No.	Hen's diet	No of eggs analyzed	Egg weight	Cystine per egg	Methionine per egg
			gm.	mg.	mg.
1	Low protein	16	59 ±0.48	138 ±4.2	195 ±2.7
	High " (casein)	21	+60.9 ±0.4	+181 ±2.6	+206 ±1.9
	" " " + 0.5 gm. methionine	4	61.0	175	207
	High protein (soy bean)	18	57.5 ±0.89	144 ±4.7	-171 ±4.6
	" " (" " + 0.2 gm. methionine)	7	57.9 ±5.1	143 ±6.9	166 ±6.9
	High protein (gelatin)	8	56.4 ±1.2	155 ±4.1	186 ±5.6
	Low " "	16	60.3 ±2.88	158 ±1.41	192 ±1.43
2	High " (casein)	23	62.0 ±0.6	+185 ±2.04	+234 ±3.93
	" " (gelatin)	5	-52.1 ±1.5	142 ±5.1	-158 ±11.1
	" " (soy bean + 0.1 gm. methionine)	10	60.2 ±0.96	161 ±4.0	197 ±7.2
	High protein (soy bean)	12	61.1 ±0.49	162 ±3.8	196 ±6.0

* The bold-faced figures are statistically significant increases (+) or decreases (-).

When the high protein diet containing 20 per cent casein was fed, the average methionine and cystine content and the weights of the eggs were higher than those obtained on the low protein diet. Statistical analysis of the data showed these increases to be highly significant. Comparison of the results obtained by feeding the high protein diets containing 50 per cent soy bean meal or 20 per cent gelatin with those obtained from the low protein diet indicates a tendency for the egg weight and the methionine content to decrease. The 20 per cent casein diet gave higher cystine values in the eggs than did a 50 per cent soy bean meal diet; yet the soy bean diet

contained approximately 2 times as much cystine as did the casein. The most plausible explanation for this increase in the cystine content of the eggs is that cystine is synthesized from the other components in the diet, especially methionine. Supplementing the high protein diets that contained casein or soy bean meal with synthetic methionine did not materially affect the methionine and cystine content of the eggs. Daily methionine addition to the hen's diet in quantities of 0.5 gm. and over had a tendency to stop egg production (see gelatin feeding, Fig. 1). When we obtained eggs in isolated instances, after supplementation with 1 gm. of methionine on 3 successive days, the methionine and cystine content of the eggs did not differ from those laid previous to the methionine addition. In order to increase the levels of these two amino acids in the egg, the methionine must be in the hen's diet in sufficient quantity and in peptide form. The difference in the rate of absorption between the free and bound amino acid, which later undergoes cleavage, may be the explanation for this finding.

We think that the cystine and methionine content of an egg cannot be considered constant and may depend upon the hen's dietary protein.

The experiments offered above reveal the importance of the quantity and quality of the protein in the feed as a determining factor in establishing high levels of these sulfur-containing amino acids in the egg.

SUMMARY

Fluctuations were observed in the methionine and cystine content of the hen's eggs laid in consecutive order by hens which were kept either on a low or a high protein diet. These interim changes are, however, normal physiological variations.

Statistically significant increases were established in cystine and methionine values when a low protein diet was changed to a high by supplementation with casein (20 per cent).

Feeding synthetic methionine as a supplement did not affect the level of cystine and methionine content of the egg. Apparently, to increase the levels of these two amino acids in the egg, sufficient methionine must be in the hen's diet in peptide form.

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THE ACTION OF *ESCHERICHIA COLI* ON ACETYLDEHYDROAMINO ACIDS*

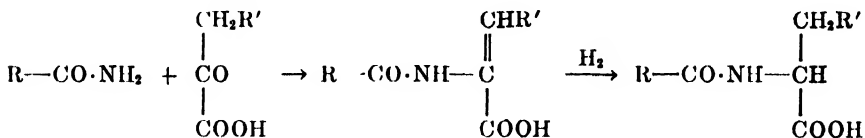
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The possible rôle of the α,β -unsaturated amino acids in the intermediary metabolism of the amino acids has received recurrent attention since Dakin (1) and Bergmann (2) suggested that the biochemical dehydrogenation of amino acids might involve the formation of derivatives of aminoacrylic acid (dehydroalanine). Direct evidence for this hypothesis has proved to be elusive thus far, since, even if formed, the dehydroamino acids may be expected to undergo rapid hydrolysis with the formation of the corresponding keto acid and ammonia. The acylated dehydroamino acids, however, are stable compounds (2-6) and lend themselves to studies on their possible participation in enzyme-catalyzed metabolic reactions. Thus, Bergmann and Schleich (7) discovered an enzyme (dehydropeptidase) specifically adapted to the hydrolysis of peptide bonds in compounds such as glycyldehydroalanine or glycyldehydrophenylalanine. Although the physiological rôle of dehydropeptidase remains obscure, its presence in numerous animal tissues (8) gives support to the view that the dehydroamino acids, or their derivatives, represent active participants in intermediary protein metabolism.

A number of years ago it was suggested (9) that there may exist metabolic analogues to the chemical reactions between keto acids and acid amides, described by Bergmann and Grafe (4) and later studied by Shemin and Herbst (10). Such reactions lead to the formation of acyldehydroamino acids, according to the following scheme.



If Group R in the above reaction sequence is considered to represent the remainder of an amino acid residue, the biological coupling between condensation and reductive reactions of this type would offer a metabolic pathway for the synthesis of peptides (9). The occurrence of enzyme-

* These studies were aided by grants from the Rockefeller Foundation, the James Hudson Brown Fund of the Yale School of Medicine, and the Williams-Waterman Fund of the Research Corporation.

catalyzed reactions between amino acid amides and keto acids has been postulated recently (11) in order to explain the effect of pyruvic acid in accelerating the desamidation of glutamine and asparagine by liver extracts.

In the present communication, additional evidence is offered for the possible participation of acylated dehydroamino acids in metabolic reactions. The data presented in this paper show that several acetyldehydroamino acids are metabolized by growing cultures of *Escherichia coli*.

Absorption Spectra of Acetyldehydroamino Acids—The study of the biochemical transformation of the derivatives of acrylic acid is notably facilitated by the characteristic ultraviolet absorption spectra of this group of compounds. Thus, Warburg and Christian (12) were able to employ the selective absorption at 2400 Å of phosphoenolpyruvic acid as an index of the formation or disappearance of this substance in the presence of enolase. Similarly, Carter and Greenstein (13) have followed the action of dehydropeptidase on glycyldehydroalanine by means of a spectrophotometric method in which there is measured the rate of the disappearance of the absorption at 2400 Å.

For the spectrophotometric study of the biochemical transformation of derivatives of aminoacrylic acid, the aromatic members of this group are especially suitable. As is well known, tyrosine exhibits characteristic absorption bands in the near ultraviolet region of the spectrum. The position in the wave-length scale and the magnitude of the maxima of these bands depend on several factors, notably the pH of the solution (14). However, when α,β -unsaturation is present in an acyl derivative of one of these aromatic amino acids, the observed absorption spectrum is markedly different from that for the parent amino acid derivative. This is illustrated in Fig. 1, where the absorption spectra of acetyldehydrotyrosine and acetyltyrosine are compared in both acid and alkaline solutions. As in the case of the tyrosine derivatives, the comparable derivatives of phenylalanine and dehydrophenylalanine show appreciable differences in the position and magnitude of their absorption maxima (cf. Table I). The marked increase in the molecular extinction coefficient and the shift to a longer wave-length, caused by the introduction of a double bond in conjugation with the benzene ring, may be attributed to the greater resonance of the dehydro compound, as compared with the parent acylamino acid.

The intense absorption of the aromatic acyldehydroamino acids in the near ultraviolet makes them valuable for biochemical studies, especially in the presence of tissue extracts which absorb strongly at wave-lengths near 2400 Å, where the aliphatic dehydroamino acid derivatives show selective absorption. For this reason, studies on the purification and properties of dehydropeptidase, which have been under way in this labora-

tory,¹ have involved the use of glycyldehydrophenylalanine as the substrate.

In Fig. 1, the absorption spectra of *p*-hydroxyphenylpyruvic acid are included. The coincidence in position on the wave-length scale of the absorption maximum of the keto acid at pH 2 with that of acetyltyrosine, and at pH 11 with that of acetyldehydrotyrosine, may be taken as an expression of the pronounced enolization of the keto group in alkaline solution.

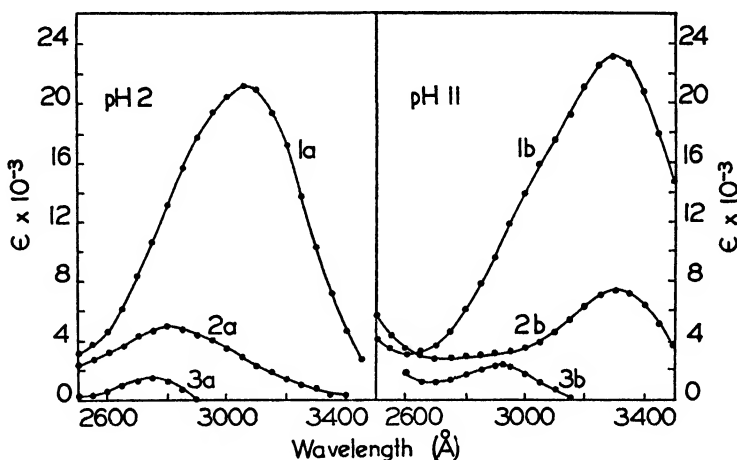


FIG. 1. Absorption spectra of acetyldehydrotyrosine and related compounds in acid (pH 2) and alkaline (pH 11) solution. Curves 1, *a* and 1, *b*, acetyldehydrotyrosine; Curves 2, *a* and 2, *b*, *p*-hydroxyphenylpyruvic acid; Curves 3, *a* and 3, *b*, acetyl-L-tyrosine.

The position and magnitude of the maximum extinction coefficients of a number of acetyldehydroamino acids and their derivatives are given in Table I, and provide the basis for the quantitative data which are presented in what follows.

Transformation of Acetyldehydrotyrosine by Escherichia coli—In a previous communication (18) it was shown that a *tyrosineless* mutant of *Escherichia coli* (strain 58-5030) cannot utilize acetyldehydrotyrosine or acetyl-L-tyrosine for growth on a tyrosine-deficient medium. However, when acetyldehydrotyrosine was present in a medium containing L-tyrosine, thus permitting bacterial growth, it was found that the *E. coli* caused the disappearance of the characteristic absorption of the acetyldehydroamino acid at 3050 Å. The further study of this phenomenon

¹ Yudkin, W. H., and Fruton, J. S., unpublished experiments.

involved the use of a strain of *E. coli* (K-12) which will grow on a medium containing glucose, asparagine, and salts (19).

The data in Table II show that incubation for 48 hours at 25° has resulted in almost complete disappearance of the characteristic band of acetyldehydrotyrosine in acid solution. This result was noted not only with the bacterial strains K-12 and 58-5030, but also with strain 58-278, which is a *phenylalanineless* mutant of *Escherichia coli*.

TABLE I

Molecular Extinction Coefficients of Acetyldehydroamino Acids and Related Compounds

Substance	Wave-length	$\epsilon_{\text{max.}}^*$	
		pH 2	pH 11
	<i>A</i>		
Acetyldehydrotyrosine·H ₂ O (2)†	3050	21,100	
	3300		23,050
Acetyl-L-tyrosine (15)	2750	1,505	
	2925		2,200
<i>p</i> -Hydroxyphenylpyruvic acid (16)	2800	4,900	
	3300		7,250
Acetyldehydrophenylalanine (3)	2825	15,400	
	2725		13,840
Acetyl-L-phenylalanine (15)	2575	250	240
Phenylpyruvic acid (17)	2875	1,290	
Acetyldehydroleucine (6)	2400‡	4,360	
Acetyldehydroalanine (4)	2400	4,600	

* Determined by means of a Beckman ultraviolet spectrophotometer.

† The figures in parentheses refer to bibliographic references.

‡ No distinctive absorption band was noted in the spectrum of this compound. The extinction coefficient at 2400 Å thus does not represent the value for an absorption maximum.

The question may be raised as to whether the transformation of acetyldehydrotyrosine is a property of actively growing *Escherichia coli* or whether "resting" cells are also capable of effecting this reaction. An experiment was accordingly set up in which strain K-12 was inoculated into a minimum medium and grown for 48 hours at 25°. The cells were then harvested by centrifugation, and resuspended in 10 cc. of a medium containing salts and 2 mg. of acetyldehydrotyrosine, but no sugar or asparagine. This suspension was incubated for 48 hours at 25°, together with appropriate controls containing no acetyldehydroamino acid. Acidified filtrates were prepared in the manner described in Table II and the absorption at 3050 Å was then determined. In contrast to the nearly complete disappearance of the absorption at 3050 Å observed for the acid filtrates

obtained with actively growing bacteria, only a slight decrease (12 per cent) was noted in the case of the "resting" cells. It would appear, therefore, that the conversion of the acetyldehydroamino acid is associated

TABLE II

Effect of Escherichia coli on Acetyldehydroamino Acids

Escherichia coli were inoculated into 10 cc. of a minimum medium containing 2 mg. of the acetyldehydroamino acid. In experiments with the bacterial strain 58-5030 (*tyrosineless*), 0.1 mg. of DL-tyrosine and 0.01 γ of biotin were also present, while, in the case of strain 58-278 (*phenylalanineless*), there were added 0.1 mg. of DL-phenylalanine and 0.01 γ of biotin. The tubes were incubated at 25° for 48 hours. After this time, 2 cc. of 20 per cent trichloroacetic acid were added and the suspension was filtered by gravity into a 250 cc. volumetric flask. The solid matter on the filter was washed thoroughly with water, and the combined filtrate and washings were diluted to 250 cc. Control experiments were set up in which the medium contained no acetyldehydroamino acid. These were treated as above and the filtrates were used as comparison blanks in the Beckman spectrophotometer. Further controls were run in which the media containing acetyldehydroamino acid were not inoculated with bacteria. These were incubated as above and, after acidification and dilution to 250 cc., were read in the spectrophotometer against a sample of the acidified and diluted minimum medium to which no acetyldehydroamino acid had been added.

Acetyldehydroamino acid	Inoculum	Optical density of diluted acid filtrate	
		Wave-length	Log I/I ₀
Acetyldehydrotyrosine	None	A	
		3050	0.702
"	<i>E. coli</i> (58-5030)	2750	0.352
		3050	0.021
"	" " (K-12)	2750	0.066
		3050	0.034
"	" " (58-278)	2750	0.070
		3050	0.033
Acetyldehydrophenylalanine	None	2750	0.072
		2825	0.608
"	<i>E. coli</i> (K-12)	2575	0.332
		2825	0.542
Acetyldehydroleucine	None	2575	0.316
		2400	0.194
"	<i>E. coli</i> (K-12)	2400	0.188
Acetyldehydroalanine	None	2400	0.242
		2400	0.025

with metabolic processes which proceed during the period of active bacterial growth.

That the transformation of acetyldehydrotyrosine occurs during the period of growth is further indicated by the data presented in Table III.

Comparison, at different time intervals following inoculation of the medium, of the extent of growth and the extent of conversion of acetyldehydrotyrosine shows that nearly all of the acetyldehydroamino acid has disappeared when the bacterial growth has attained a half maximum value.

Effect of Escherichia coli on Other Acetyldehydroamino Acids—It will be noted from the data presented in Table II that, in contrast to acetyldehydrotyrosine, acetyldehydrophenylalanine is metabolized only slightly by growing *Escherichia coli* under the conditions of these experiments. Under similar experimental conditions, the absorption of acetyldehydroleucine at 2400 Å is not decreased appreciably following incubation with *E. coli*, and it would appear, therefore, that this aliphatic acetyldehydro-

TABLE III

Relation of Bacterial Growth to Conversion of Acetyldehydrotyrosine by Escherichia coli

Time after inoculation hrs.	Bacterial growth		Optical density of diluted acid filtrate at 3050 Å†	Conversion of dehydro compound
	Density*	per cent		
0	0	0	0.675	0
23.66	0.0351	20	0.625	7.4
28	0.0458	26	0.553	18.1
31.66	0.0706	41	0.187	72.3
35.75	0.1079	62	0.038	94.4
48	0.1739	100	0.016	97.6

* Determined by means of an Evelyn photoelectric colorimeter.

† The diluted acid filtrates were prepared as described in Table I. Their ultra-violet absorption spectra were determined with, as comparison blanks, solutions obtained from bacterial cultures of the same age, but to which no acetyldehydrotyrosine had been added initially. The extent of bacterial growth was found to be the same in the presence or the absence of the dehydro compound. Under these experimental conditions, therefore, this substance does not exert a detectable inhibitory effect on the growth of strain K-12 of *Escherichia coli*.

amino acid is more resistant to bacterial action. On the other hand, acetyldehydroalanine is readily metabolized by growing *E. coli*, as judged by the disappearance of the band at 2100 Å.

Isolation of Metabolic Product Derived from Acetyldehydrotyrosine—The examination of the spectrophotometric data in Table II offers some information as to the possible fate of the acetyldehydrotyrosine metabolized by the growing bacteria. It will be noted that, at the end of the incubation period, the absorption at 2750 Å is notably higher than that at 3050 Å. Indeed, the complete absorption curve for the diluted acid filtrate has its maximum near 2750 Å. In alkaline solution, the maximum is shifted to 2900 Å. Qualitatively, these data suggest that acetyldehydrotyrosine

has been converted, in large part, to a derivative of tyrosine, since the maxima near 2750 Å (in acid) and near 2900 Å (in alkali) appear to be characteristic of such phenolic compounds. The presence of an appreciable quantity of *p*-hydroxyphenylpyruvic acid would appear to be ruled out by the negligible absorption at 3300 Å in alkaline solution. Therefore, if hydrolytic cleavage of acetyldehydrotyrosine to the keto acid had been effected by the bacteria, this product must have undergone further transformation.

In order to determine more definitely the fate of the acetyldehydrotyrosine, an attempt was made to isolate a distinctive metabolic product from a large scale incubation mixture. 2 liters of minimum medium containing 400 mg. of acetyldehydrotyrosine were inoculated with *Escherichia coli* (strain K-12) and incubated at 25° for 48 hours. The mixture was then acidified and concentrated to a volume of 100 cc. under reduced pressure at a bath temperature of 40–45°. The concentrate was extracted with ten successive 75 cc. portions of ethyl acetate and the combined ethyl acetate extract was evaporated to dryness. The residue was dissolved in hot water, treated with charcoal, and the partially decolorized filtrate was concentrated to a volume of 5 cc. Upon chilling this solution, 148 mg. of rod-shaped crystals were obtained which, after recrystallization from water, had a melting point of 133–134°. On analysis, the substance was found to contain 57.51 per cent carbon, 5.26 per cent hydrogen, and 6.81 per cent nitrogen. The recrystallized product, obtained in another isolation experiment by the procedure outlined above, melted at 132–133° and gave analytical values of 57.42 per cent carbon, 5.27 per cent hydrogen, and 6.78 per cent nitrogen. The sodium fusion test for the presence of sulfur or halogen proved to be negative. If it is assumed that the isolated material contains only carbon, hydrogen, nitrogen, and oxygen, the analytical data cited above agree satisfactorily with the empirical formula $C_{10}H_{11}O_4N$ (theory, carbon 57.41 per cent, hydrogen 5.31 per cent, nitrogen 6.69 per cent). Free amino groups were found to be absent as judged by the Van Slyke nitrous acid method (reaction period, 20 minutes). The absence of titratable carboxyl groups was established by titration with 0.01 N alcoholic KOH, with methyl red or brom-thymol blue as the indicator. A 1 per cent solution of the substance in water has a pH of about 4. The substance gave a red color with the Millon reagent, and a violet color with ferric chloride. A 1.2 per cent solution of the product in water failed to exhibit any optical rotation when examined in a 1 dm. polarimeter tube.

The examination of the ultraviolet absorption spectrum of an aqueous solution containing 0.055 mg. of the substance per cc. (cf. Fig. 2) showed a maximum, in acid solution (pH 2), at 2725 Å ($\log I/I_0 = 0.325$) and, in alkaline solution (pH 11), at 2875 Å ($\log I/I_0 = 0.672$). If the empirical

formula deduced from the analytical data also represents the molecular formula (mol. wt. 209.2), the values of the molecular extinction coefficients are $\epsilon_{\max.} = 1240$ (at 2725 Å in acid solution) and $\epsilon_{\max.} = 2560$ (at 2875 Å in alkaline solution).

It was found, further, that, following treatment of the product with 10 per cent hydrochloric acid at 100° for 4 hours, the neutralized solution contained no L-tyrosine, as judged by a microbiological assay with the *tyrosineless* mutant strain of *Escherichia coli* as the assay organism (18). Under similar conditions of hydrolysis, acetyl-DL-tyrosine yielded the expected amount of L-tyrosine.

It may be added that attempts to obtain, by the isolation procedure outlined above, a similar substance from cultures of *Escherichia coli* grown

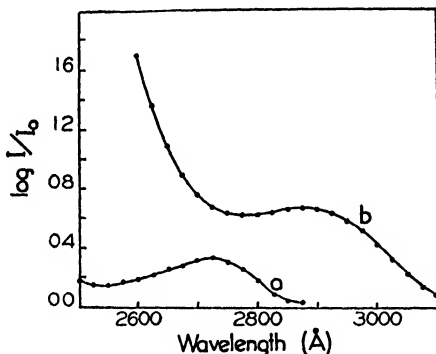


FIG. 2. Absorption spectra of the product obtained following bacterial conversion of acetyldehydrotyrosine. Concentration, 0.055 mg. per cc. Curve *a*, in acid solution (pH 2); Curve *b*, in alkaline solution (pH 11).

in the absence of acetyldehydrotyrosine were unsuccessful and, furthermore, that the concentrated aqueous solution obtained following ethyl acetate extraction showed no appreciable ultraviolet absorption in the region 2700 to 3000 Å.

Possible Nature of Metabolic Product—Since the properties of the substance and the empirical formula derived from the analytical data do not accord with those of any known simple derivative of acetyldehydrotyrosine, the elucidation of the chemical structure of the metabolic product requires further experimental work. Certain tentative conclusions as to its nature may be drawn, however. The presence in the molecule of the phenolic group is indicated by the color reactions and, more importantly, by the position and magnitude of the absorption maxima in acid and alkaline solution. Moreover, the absorption data support the idea that the α, β double bond of acetyldehydrotyrosine has disappeared, in view of

the similarity between the absorption spectra of the isolated product with those of tyrosine and its derivatives. The spectra also suggest that oxidation of the benzene ring to give a derivative of dioxyphenylalanine is unlikely, since this amino acid exhibits absorption maxima ($\epsilon_{\max.} = 2680$ at 2800 Å at pH 2; $\epsilon_{\max.} = 8650$ at 3000 Å at pH 11.5) which are rather different from those of tyrosine.

It would appear that the acetamino group of acetyldehydrotyrosine has been retained, since, upon hydrolysis with 10 per cent hydrochloric acid at 100° for 4 hours, free amino groups are liberated. Analysis of an aliquot of the hydrolysate (representing 3.66 mg. of the substance) by the Van Slyke nitrous acid method (reaction time, 20 minutes) gave 0.165 mg. of amino nitrogen. This represents 66 per cent of that to be expected (0.249 mg. of amino nitrogen). It cannot be stated at present whether the incomplete liberation of amino nitrogen is due to incomplete hydrolysis of the acetamino group or to the failure of the amino group to react quantitatively with nitrous acid during the 20 minute reaction period.

The empirical formula indicates that the isolated substance has 1 carbon atom less than acetyldehydrotyrosine, and the titration data suggest that decarboxylation has occurred. However, to account for the 2 oxygen atoms not included in the phenolic hydroxyl and the acetamino group, it seems necessary to assume that, during the bacterial conversion of acetyldehydrotyrosine there occurred oxidation of the side chain. The data secured thus far support the working hypothesis that the substance is a derivative of N-acetyltyramine in which the carbon side chain has undergone oxidation. In particular, the possibility that the substance may be a derivative of 6-hydroxyoxindole is under investigation.

Little can be said at present concerning the sequence of reactions involved in the bacterial conversion of acetyldehydrotyrosine. It would appear, however, that acetyl-L-tyrosine is not an intermediate in this process, since, following incubation of acetyltyrosine with growing *Escherichia coli* (strain K-12), the medium contains, after acid hydrolysis, the expected amount of L-tyrosine (based on a microbiological assay with the tyrosineless mutant of *Escherichia coli*). Prior to acid hydrolysis, no growth-promoting activity can be detected (*cf.* (18)). Thus, acetyltyrosine seems to be more resistant to the action of the growing bacteria than is the corresponding dehydro compound, and it is unlikely, therefore, that the bacterial conversion of acetyldehydrotyrosine involved the direct reduction of the α,β double bond.

SUMMARY

Growing cultures of *Escherichia coli* effect the transformation of several acyldehydroamino acids, with the resultant disappearance of the charac-

teristic selective absorption of these substances in the ultraviolet region of the spectrum. In the case of the bacterial conversion of acetyldehydrotyrosine, there appears an appreciable quantity of a nitrogenous phenol whose structure remains to be elucidated.

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THE EFFECTS OF CYTOCHROME^c ON ANOXIC AND CYANIDE-POISONED RATS

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Proger and his coworkers have reported that intravenously administered cytochrome *c* is capable of mitigating the physiological effects of anoxia (1-5). In view of these reports it was thought that cytochrome *c* might be of value in preventing cyanide intoxication. Albaum, Tepperman, and Bodansky (6) showed that sodium cyanide (5 mg. per kilo) injected intraperitoneally produced a 50 per cent inhibition of rat brain cytochrome oxidase. Increasing the intracellular concentration of cytochrome *c* might, conceivably, raise the level of respiration through the uninhibited fraction of the cytochrome oxidase in animals so poisoned. Experiments designed with this idea in mind failed to demonstrate, as will be shown below, any effect of cytochrome *c* in cyanide-poisoned rats. Further work was then carried out in an attempt to confirm two findings which Proger and his group had reported, which were susceptible of objective and quantitative measurement. These were the effects of cytochrome *c* on the organ content of easily hydrolyzable phosphorus and the survival times of anoxic rats.

The resynthesis of adenosine tri- and diphosphate from adenylic acid and inorganic phosphate is coupled to oxidative processes (7) and should be diminished under conditions of anoxia. The organ content of adenosine tri- and diphosphate parallels the content of easily hydrolyzable phosphorus (7-9). Further, under conditions of anoxia the blood level of lactic acid increases (10). In our experiments the amount of easily hydrolyzable phosphorus of heart and kidney, the blood level of lactic acid, and the measurement of survival times were used to determine the effect of anoxia on rats and to measure the value, if any, which cytochrome *c* has in the treatment of anoxia.

Materials and Methods

Rats—Wistar strain white rats were used. These were maintained on a diet of Gaines' dog "krunchon" supplemented by meat, fruit, vegetables, milk, and cod liver oil.

Phosphorus—Easily hydrolyzable phosphorus was determined by measuring the amount of inorganic phosphate released after 7 minutes hydrolysis at 100° in 1 *N* sulfuric acid. Analyses, before and after hydrolysis, were carried out by the method of Fiske and Subbarow (11). All analyses were performed in duplicate and the average difference between duplicates was 0.7 per cent, with a standard deviation of 1.36 per cent.

Lactic Acid—Lactic acid was determined by the method of Barker and Summerson (12), modified to the extent that the concentrated sulfuric acid was added, with mechanical stirring, to the sample in an ice bath and the copper-calcium precipitation was eliminated.

The average difference between duplicates was 1.5 per cent, with a standard deviation of 2.2 per cent. The use of mechanical stirring in place of hand shaking made the procedure much less tedious and gave more uniform mixing. The mean value for blood lactic acid found in five normal rats, 3 months old, was 30.8 mg. per cent when determined directly on the trichloroacetic acid filtrate and 30.4 mg. per cent after copper-calcium precipitation.

Cytochrome *c*—Cytochrome *c* was prepared according to the method of Keilin and Hartree (13) from beef hearts. The dialyzed solution was at first kept at 4° without a preservative. Bacterial growth was soon noted and the cytochrome *c* was repurified, dried from the frozen state *in vacuo*, and kept at 4°. The enzymatic activity was tested according to the procedure used by Keilin and Hartree (13), with both ascorbic and succinic acids as substrates and rat brain homogenate as a source of cytochrome oxidase. With ascorbic acid as substrate the cytochrome *c* preparation showed an average oxygen uptake of 263 c.mm. of oxygen per hour per mg. of cytochrome *c* under the following conditions: 38°, pH 7.4, total volume of reactants 3.0 ml., containing 29.2 mg. of fresh homogenized rat brain. Under the same conditions, but with succinic acid as substrate, the average oxygen uptake was 101 c.mm. of oxygen per hour per mg. of cytochrome *c*.

The purity of the cytochrome *c* preparation was tested with a Beckman photoelectric spectrophotometer and the extinction coefficients given by Rosenthal and Drabkin (14). The value for the ratio of the extinction at 550 $m\mu$ to that at 535 $m\mu$ for reduced cytochrome *c* in 0.2 *N* sodium hydroxide was 3.86. Rosenthal and Drabkin reported 3.89 for this ratio. The concentrations of all cytochrome solutions used were determined by spectrophotometric analyses. In converting spectrophotometric values to a weight basis, 16,500 was used as the molecular weight of cytochrome *c*.

Iron analysis of the cytochrome *c* preparation, after repurification, by the method of Drabkin (15) and also by sulfuric acid-hydrogen peroxide digestion, gave an iron content of 0.349 per cent.

An aqueous solution of the cytochrome *c* preparation containing approximately 0.5 per cent sodium chloride was used for injection.

Oxygen—Cylinders of 3 per cent oxygen-97 per cent nitrogen were used (Matheson). Analysis of the gas in the Van Slyke manometric apparatus (16) gave values of 3.9 per cent oxygen and 96.1 per cent nitrogen. The gas present in the liter bottle after a rat had been in it for 8 minutes, under conditions of flow used in the experiments, was also analyzed. The averages of three analyses were 3.5 per cent oxygen, 0.9 per cent carbon dioxide, and 95.6 per cent nitrogen.

For the survival experiments a lower percentage of oxygen was needed, and this was obtained by diluting 7 parts of the 3.9 per cent oxygen with 2 parts of 100 per cent nitrogen. The mixture was made by means of the flowmeters removed from a "midget kinet-o-meter" anesthesia machine (Ohio Chemical and Manufacturing Company). This mixture, analyzed in the Van Slyke manometric apparatus, consisted of 2.8 per cent oxygen and 97.1 per cent nitrogen.

Respirator—The respirator (Marco air pump, model B2, J. B. Maris Company, Bloomfield, New Jersey) delivered gas at a pressure of 80 mm. of water through a tracheal cannula, and was run at 100 cycles per minute. Expiration was passive.

Trichloroacetic Acid—Baker's c.p., analyzed material was used. 5 per cent solutions were made up freshly each morning and kept in the refrigerator until used.

Sodium Pentobarbital (Nembutal, Abbott)—A solution containing 60 mg. per ml. of nembutal in 10 per cent alcohol, marketed as "veterinary nembutal," was used. A dosage of 45 mg. per kilo was given intraperitoneally.

Sodium Cyanide—Baker's c.p. material was used. Titration against silver nitrate showed it to be 95.7 per cent sodium cyanide. The dosage was always calculated in terms of cyanide ion and solutions for use were made freshly each day.

EXPERIMENTAL

Cytochrome c and Cyanide

Experiment I. Nembutal Anesthesia; Easily Hydrolyzable Phosphorus and Lactate—Paired, litter mate white rats of the same sex and between 4 and 6 months old were used in this experiment. Each rat of a pair was anesthetized with nembutal and, after 12 minutes, one (test) was given cytochrome *c* (20 mg. per kilo; solution containing 10 mg. per ml.), and the other (control) was given an equal volume of 0.85 per cent sodium chloride solution, by injection. With the exception of the nembutal, all injections in this and subsequent experiments were made into the femoral vein. 5 minutes after the injections intratracheal artificial respiration with air was started. This was necessary because injections of sodium cyanide were

followed almost immediately by a brief tachypnea, and then by a period of apnea lasting 1 or 2 minutes. Administration of 5 per cent carbon dioxide without artificial respiration did not prevent the apnea. Artificial respiration made it possible to assume that the effects measured were due to cyanide and not to anoxia. 10 minutes after the cytochrome *c* or saline injections sodium cyanide (1.1 mg. of cyanide per kilo; solution containing 2.2 mg. of cyanide per ml.) was injected intravenously over a

TABLE I

Effect of Cyanide on Blood Lactate and 7 Minute Kidney and Heart Phosphorus in Rats with and without Previously Injected Cytochrome c

Each set of rats was of the same litter and sex. Group 1 was given 2 ml. per kilo of 0.85 per cent sodium chloride solution; Group 2, 20 mg. per kilo of cytochrome *c* (10 mg. per ml.). All rats received 1 mg. per kilo of CN (2.2 mg. per ml.). Correlation of litter mates was taken into account in the test of significance.

Set No.	Weight of rats		Sex	Blood lactate per 100 ml.		Kidney 7 min. P per 100 gm. fresh tissue		Heart 7 min. P per 100 gm. fresh tissue	
	Group 1	Group 2		Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
	gm.	gm.		mg.	mg.	mg.	mg.	mg.	mg.
1	277	292	M.	58.0	45.4	8.3	6.5	16.7	15.6
2	363	310	"	44.3	62.7	7.3	9.8	18.7	14.5
3	345	377	"	39.1	76.3	10.1	9.0	22.4	15.3
4	324	308	"	59.3	63.4	9.3	12.3	19.2	17.9
5	230	215	F.	97.6	101.0	8.1	8.5	18.6	11.8
6	223	235	"	101.0	63.6	9.6	10.2	16.2	16.2
7	303	316	M.	54.7	54.5	6.3	8.8	13.0	14.1
8	227	208	F.	49.5	85.2	4.4	3.6	11.3	9.1
9	373	355	M.	37.6	68.4	8.6	7.4	18.4	14.3
10	335	330	"	84.8	46.5	7.6	7.5	10.9	16.1
11	275	270	"	60.2	47.6	8.6	8.1	15.4	17.9
12	290	282	"	63.9	64.8				
Mean of differences, Groups 2 and 1				2.45		0.32		-1.64	
P				0.74		0.53		0.18	

10 minute period. During the infusion the abdomen was opened. Immediately on completion of the cyanide injection, the left kidney was clamped, excised, and weighed on a torsion balance and then homogenized in 100 ml. of cold 5 per cent trichloroacetic acid in a Waring blender for 2 minutes. The interval from the time the kidney was clamped until it was placed in acid was always 30 seconds. 0.5 ml. of blood was taken from the heart and delivered into 50 ml. of 5 per cent trichloroacetic acid. The heart was then excised, weighed, and placed in 100 ml. of cold 5 per cent tri-

chloroacetic acid in the Waring blender $1\frac{1}{2}$ minutes after the kidney had been clamped, and homogenized for 2 minutes. The heart and kidney homogenates were filtered, and the filtrates analyzed for easily hydrolyzable phosphorus; the filtrate of the blood was analyzed for lactate.

It was necessary to control the time intervals at which the organs were removed and the trichloroacetic acid homogenates prepared, because the labile phosphorus compounds are unstable and appreciable breakdown can be detected in dog brain within 5 seconds of its removal from the animal (8).

Results The values found in this experiment are presented in Table I. The differences between the results on the test and control animal of each pair of litter mates were found, and from these differences the value of Fisher's *t* and the corresponding value of *P* (17), representing the probability that the observed differences were due to chance, were determined. The probabilities in Table I indicate that there are no significant differences between those members of the pairs given cytochrome *c* and those given saline.

For comparison, the average values of easily hydrolyzable phosphorus of kidney and heart and of blood lactate in six rats treated exactly as the control, except that they were given saline in place of cyanide as well as in place of cytochrome *c*, were as follows: kidney 7 minute phosphorus, 11.0 mg. per 100 gm. of fresh tissue; heart 7 minute phosphorus, 21.4 mg. per 100 gm. of fresh tissue; blood lactate, 23.1 mg. per 100 ml. of blood.

With the dose of cyanide given none of the animals died before the heart was excised, although the heart rate dropped from about 400 to about 150 per minute during the cyanide infusion.

Cytochrome c and Low Oxygen Mixtures

Experiment II. Nembutal Anesthesia; Easily Hydrolyzable Phosphorus and Lactate—This was similar to Experiment I, but cyanide was omitted and artificial respiration with 3.9 per cent oxygen was given for 5 minutes instead. Triplet litter mate rats of the same sex, between 2 and 4 months old, were used. One rat of each set (designated as Group 1 in the tables) served as the control and was given 0.85 per cent saline and then artificial respiration with air. The second rat (Group 2) was given 0.85 per cent saline and subjected to artificial respiration with 3.9 per cent oxygen. The third rat (Group 3) received cytochrome *c* (22.8 mg. per kilo; solution containing 11.4 mg. per ml.) and was subjected to artificial respiration with 3.9 per cent oxygen. The saline or cytochrome *c* was given 12 minutes after the nembutal, and artificial respiration with air or 3.9 per cent oxygen was begun 10 minutes after the saline or cytochrome *c* injection. The volume of the saline or cytochrome *c* solution injected was

2.0 ml. per kilo. Tissue homogenates were prepared according to the same schedule that was used in Experiment I.

Results—The results are shown in Table II. Differences between the values found in Groups 1 and 2 and the corresponding *P* values show a highly significant difference between the two populations, indicating that

TABLE II

Effect of Exposure to 3.9 Per Cent Oxygen on Blood Lactate and 7 Minute Kidney and Heart Phosphorus in Rats with and without Previously Injected Cytochrome c (Nembutal Anesthesia)

Each set of rats was of the same litter and sex. Group 1 was pretreated with saline, then given artificial respiration with air; Group 2 was pretreated with saline, then given artificial respiration with 3.9 per cent oxygen; Group 3 was pretreated with cytochrome *c*, then given artificial respiration with 3.9 per cent oxygen. Correlation of litter mates was taken into account in the test of significance.

Set No.	Rat weight			Sex	Blood lactate per 100 ml.			Kidney 7 min P per 100 gm. fresh tissue			Heart 7 min P per 100 gm. fresh tissue		
	Group 1	Group 2	Group 3		Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
	gm.	gm.	gm.		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	295	304	278	M.	27.7	66.4	75.4	9.9	8.9	8.0	21.5	16.0	19.2
2	245	245	227	"	23.3	84.2	66.3	11.1	8.1	9.7	19.4	16.3	16.7
3	185	181	186	F.	28.0	119.8	107.4	11.0	7.9	7.0	22.0	15.3	18.9
4	264	278	256	M.	24.4	91.4	92.8	10.5	10.4	10.4	20.1	19.3	18.2
5	163	160	168	F.	30.9	70.8	92.4	12.3	11.9	7.5	16.5	13.0	14.0
6	292	299	303	M.	22.7	83.2	97.5	8.6	5.8	5.9	18.3	14.6	15.3
7	222	252	268	"	39.9	58.1	81.8	10.1	7.6	6.1	19.8	12.2	11.4
8	257	254	282	"	18.4	79.6	83.5	9.9	9.4	7.4	22.8	22.2	19.0
9	150	158	168	F.	15.1	46.1	32.0	13.7	9.9	9.9	23.1	17.1	14.9
10	152	161	155	"	15.6	44.8	45.6	12.7	11.7	10.2	22.8	19.6	18.7
Mean of differences, Groups 2 and 1					49.8			-1.72			-4.07		
<i>P</i>					<0.01			<0.01			<0.01		
Mean of differences, Groups 3 and 2					2.91			-0.95			0.07		
<i>P</i>					0.53			0.10			>0.90		

the effects of the low oxygen mixture on the phosphorus and lactate values are real. Differences between values found in Groups 2 and 3 give *P* values showing no significant difference between these members of the set, indicating that there is no evidence that cytochrome *c* is more effective in preventing the measured effects of 3.9 per cent oxygen than is saline.

Experiment III. Ether Anesthesia; Easily Hydrolyzable Phosphorus and Lactate—This experiment was performed to eliminate the possibility

that the nembutal may have interfered with oxygen utilization by the rat. Jowett and Quastel (18) have shown that the oxygen uptake of rat brain slices is diminished by about 10 per cent when phenobarbital is present in concentrations roughly equivalent to that necessary to produce narcosis *in vivo*. The same workers reported (19) that ether, in con-

TABLE III

Effect of Exposure to 3.9 Per Cent Oxygen on Blood Lactate and 7 Minute Kidney and Heart Phosphorus in Rats with and without Previously Injected Cytochrome c (Ether Anesthesia)

Each set of rats was of the same litter and sex. Group 1 was pretreated with saline, and remained in air; Group 2 was pretreated with saline, then placed in 3.9 per cent oxygen; and Group 3 was pretreated with cytochrome *c*, then placed in 3.9 per cent oxygen. Correlation of litter mates was taken into account in the test of significance.

Set No	Rat weight			Sex	Blood lactate per 100 ml.			Kidney 7 min. P per 100 gm. fresh tissue			Heart 7 min. P per 100 gm. fresh tissue		
	Group 1	Group 2	Group 3		Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
	gm.	gm.	gm.		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	218	211	231	M.	18.4	140.0	133.0	7.6	7.2	4.6	21.1	16.6	16.2
2	215	185	174	"	39.2	120.0	126.0	7.7	5.9	3.9	19.8	11.0	13.4
3	215	217	215	"	35.0	152.0	185.0	6.6	2.8	3.4	17.7	12.3	11.0
4	150	166	166	F.	43.9	141.0	136.0	9.0	7.5	7.8	19.9	17.3	14.7
5		143	130	"		128.0	141.0		5.7	7.2		13.1	9.1
6		170	168	"		188.0	161.0		6.9	4.7		14.9	14.9
7	172	144	148	"	39.6	135.0	174.0	6.5	3.7	4.0	19.6	21.4	17.4
8	246	205	194	M.	24.4	135.0	137.0	5.5	5.3	3.8	20.5	16.6	13.7
9		155	166	"		126.0	104.0		4.5	7.3		16.6	13.1
10		165	163	F.		184.0	164.0		3.8	6.6		14.6	17.3
11	171	170	165	"	52.8	206.0	187.0	9.1	3.0	6.0	21.2	9.1	14.1
Mean of differences, Groups 2 and 1					110.8			-2.37			-5.07		
<i>P</i>					<0.01			0.025			0.025		
Mean of differences, Groups 3 and 2					-0.64			0.27			-0.78		
<i>P</i>					>0.90			0.68			0.41		

centrations similar to those found in the blood in surgical anesthesia, does not significantly impair the oxygen uptake of rat brain or liver slices. In this experiment the rats were anesthetized only for the period required to give the saline or cytochrome *c* injections. Artificial respiration was not used.

Sets of triplet litter mate rats of the same sex, fasted overnight, were used. These rats were about 3 months old. The first rat (Group 1)

was given 0.5 ml. of saline and permitted to breathe air. The second rat (Group 2) received 0.5 ml. of saline, and after 10 minutes was placed in a jar containing 3.9 per cent oxygen with the gas flowing through the jar at 300 ml. per minute. The third rat (Group 3) was given 0.5 ml. of saline containing 5.7 mg. of cytochrome *c* and placed in the jar containing 3.9 per cent oxygen after 10 minutes. 18 minutes after the saline or cyto-

TABLE IV

Survival Time of Rats in 2.8 Per Cent Oxygen with and without Previously Injected Cytochrome c

Group 1 was pretreated with saline; Group 2 was pretreated with cytochrome *c*; both rats were placed in 2.8 per cent oxygen simultaneously. Correlation between members of each set due to identity of sex and similarity in weight and age taken into account in test of significance.

Set No	Rat weight		Sex	Survival time		Set No	Rat weight		Sex	Survival time	
	Group 1	Group 2		Group 1	Group 2		Group 1	Group 2		Group 1	Group 2
	gm.	gm		min	min		gm	gm.		min.	min.
1	84	84	M.	11.70	12.48	14	138	139	F.	8.05	11.32
2	122	107	"	14.18	9.58	15	182	152	"	13.42	17.37
3	91	105	"	18.92	24.90	16	131	132	"	9.77	19.28
4	102	82	F.	7.00	3.93	17	102	92	M.	9.57	7.58
5	83	84	"	6.75	8.75	18	128	128	F.	11.00	5.03
6	102	99	"	6.55	11.73	19	132	126	"	10.45	8.42
7	84	90	"	12.37	15.58	20	72	106	"	6.88	15.35
8	78	78	M.	12.18	13.80	21	222	220	M.	7.15	5.30
9	119	112	"	5.50	6.70	22	218	222	"	17.12	15.22
10	55	60	"	10.55	1.40	23	212	210	"	10.70	12.35
11	95	88	"	8.17	7.13	24	215	240	"	13.93	6.93
12	64	69	"	9.30	13.42	25	207	196	"	7.50	9.00
13	100	82	"	6.13	5.87						
Mean of differences between Groups 2 and 1										0.66	
<i>P</i>										0.45	

chrome *c* injections, the rats were removed from the jar and 5 seconds later were decapitated. Blood was collected in a beaker containing heparin. The heart was excised, weighed, and placed in 100 ml. of cold 5 per cent trichloroacetic acid 55 seconds after decapitation. The kidney was in acid $1\frac{1}{2}$ minutes after decapitation. The preparation of the filtrates and the analyses were performed as in the previous experiments.

Results The results of the experiment, given in Table III, again show that, although a significant change in the easily hydrolyzable phosphorus and lactate values was produced by the 3.9 per cent oxygen, cytochrome *c* was no more effective than saline in preventing these changes.

Experiment IV. Survival Time of Rats in Low Oxygen with and without Cytochrome c—This experiment was designed to determine whether rats given cytochrome *c* could survive in an atmosphere of low oxygen content longer than rats given saline. A mixture containing 2.8 per cent oxygen was used because the survival time of rats varied too greatly (8 to 420 minutes) in 3.9 per cent oxygen.

Paired rats of the same sex, age, and approximate weight, but not always from the same litter, were used in each experiment; they were from 1 to 4 months old. A pair of rats was anesthetized with ether as in Experiment III. One was injected with 0.5 ml. of saline and the other with an equal volume of solution containing 5.7 mg. of cytochrome *c*. 10 minutes after injection both rats were placed in a 5 pint jar through which 2.8 per cent oxygen was passed at 900 ml. per minute. The survival time of each rat was recorded to the point where respiration ceased.

Results—Table IV gives the results of this experiment. The *P* value indicates that there is no significant difference in survival times.

Excretion of Cytochrome c

Experiment V—Four rats were anesthetized with nembutal and each was injected first with 5.7 mg. per kilo of cytochrome *c* contained in 0.5 ml. of saline, and then with 2 ml. of saline. Five rats were similarly treated but given only 1.14 mg. per kilo of cytochrome *c*. The urethrae were clamped, and 3½ to 4 hours later urine was aspirated from the bladder. The urine was treated by the method Rosenthal and Drabkin (14) had applied to tissues, and the cytochrome *c* determined spectrophotometrically. Control solutions of urine containing cytochrome *c* alone, hemoglobin alone, and cytochrome *c* and hemoglobin were also treated in the same manner.

Results—In the rats given 5.7 mg. per kilo of cytochrome *c*, an average of 5.4 per cent (range, 1.8 to 6.8 per cent) of the amount administered was recovered in the urine. In the combined urine samples of five rats, each given 1.14 mg. per kilo of cytochrome *c*, no cytochrome *c* could be detected.

Attempts at recovery of similar quantities of cytochrome *c* resulted in approximately 50 per cent recovery in the presence of urine. Hemoglobin did not interfere and was completely removed. The losses apparently occurred during filtration.

Conclusions

The results of this investigation indicate that cytochrome *c*, administered intravenously, does not materially affect the lowering of the easily hydrolyzable phosphorus in kidney and heart or elevation of blood lactate brought about by the administration of cyanide. They also give no indication that similar effects produced by anoxia are prevented by cytochrome

c, or that the survival time of rats in an anoxic atmosphere is affected by administration of this enzyme. Finally, it has been shown that appreciable amounts of cytochrome *c* can be detected in the urine of rats given 5.7 mg. per kilo intravenously.

Those results obtained in this investigation which pertain to the effects of low oxygen mixtures do not confirm those reported by Proger (1, 3, 5).

SUMMARY

1. Cytochrome *c*, given intravenously, shows no effect on the decrease in the easily hydrolyzable phosphorus of kidney and heart or on the increase in blood lactate produced in rats by injection of sodium cyanide.

2. Cytochrome *c*, given intravenously, shows no effect on the decrease in the easily hydrolyzable phosphorus of kidney and heart or on the increase in blood lactate produced in rats by exposure to 3.9 per cent oxygen.

3. Cytochrome *c*, given intravenously, shows no effect on the survival times of rats placed in an atmosphere containing 2.8 per cent oxygen.

4. Approximately 5 per cent of an intravenous dose of 5.7 mg. of cytochrome *c* per kilo can be recovered in the urine; none is spectrophotometrically detectable after an intravenous injection of 1.14 mg. per kilo.

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FRACTIONATION OF GROWTH-STIMULATING FACTOR IN LIVER*

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In a previous paper (1) results were presented to show that an alcoholic extract of liver produced a pronounced growth stimulation in rats fed a natural diet. The results showed that the activity was not due to any of the known vitamins and that yeast did not possess similar activity.

Bosshardt and collaborators have described a growth-stimulating effect of liver in rats (2), while Zucker and Zucker (3) described a syndrome involving kidney lesions in rats which was cured with liver. Liver has been shown to contain factors essential for the nutrition of the monkey (4) and for the growth stimulation of *Streptococcus faecalis* (5).

The present investigation was undertaken to improve the assay procedure with rats and to prepare liver fractions which would show higher activity.

EXPERIMENTAL

All the experiments were made with male Sprague-Dawley rats 35 to 45 gm. in weight. Each animal was kept in a separate cage, and food and water were given *ad libitum*.

The ration used consisted of whole ground yellow corn 46.35 per cent, commercial soy bean meal 46.35 per cent, corn oil (Mazola) 5 per cent, cystine 0.3 per cent, CaHPO_4 0.92 per cent, CaCO_3 0.60 per cent, NaCl (iodized) 0.44 per cent, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.04 per cent.

To each kilo of this ration the following amounts of vitamins were added: thiamine 3 mg., calcium pantothenate 20 mg., pyridoxine hydrochloride 2 mg., choline hydrochloride 1 gm., nicotinic acid 20 mg., folic acid 0.25 mg., biotin 0.1 mg., inositol 100 mg., *p*-aminobenzoic acid 250 mg. 1 drop of oleum percomorphum diluted with 3 parts of corn oil was fed once weekly

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by dropper. The level of nicotinic acid was high enough to counteract the growth-inhibiting effect of corn found by Krehl *et al.* (6).

A comparison of the growth responses of rats on this ration and on a synthetic ration with and without liver supplementation is given in Table I. The synthetic ration contained sucrose 73, casein (Smaco) 18, corn oil (Mazola) 5, Salts IV 4 per cent, and the same vitamin supplements as used in the natural diet. The rate of growth for the animals on the supplemented natural ration was greater than for those on the supplemented synthetic ration, although the rate of growth for the rats on the two unsupplemented diets was about the same. Consequently, the corn-soy bean meal diet was used in all of the assays.

Extracts of fresh liver were prepared as follows: 1 kilo of fresh beef liver was ground in a meat grinder and mixed with 2 liters of 95 per cent alcohol.

TABLE I

Comparison of Growth Response of Rats to Liver Extract When Fed Synthetic Ration Or Soy Bean Meal-Corn Ration*

Group	No of animals	Diet	Supplement	Average weekly weight gain in 5 wks
A	6	Synthetic		25.8
B	6	"	Liver extract	30.0
C	12	Soy meal corn ration		23.5
D	12	" " " "	Liver extract	33.1

* 3 drops of an alcoholic extract of fresh liver equivalent to 0.45 gm. of dried fresh liver were fed daily by dropper.

The mixture was filtered through cheese-cloth and the material remaining in the cloth was pressed in a filter press. The residue was treated twice with 1 liter of 60 per cent alcohol, filtered, and pressed. The combined filtrates were distilled under reduced pressure to a volume of 100 ml., extracted three times with ether, and filtered. Traces of ether were removed by distillation under reduced pressure. 1 ml. of this extract was equivalent to 10 gm. of fresh liver. In the preliminary experiments, each rat received 3 drops of this preparation daily by dropper. This intake was equal to 1.5 gm. of fresh liver or 0.45 gm. of dried fresh liver per day.

Extracts of the dry liver powders were made by mixing 1 part of the powder with 4 parts of water and adding sufficient 95 per cent alcohol to give a 60 per cent alcohol solution. The mixture was stirred for 1 hour and placed in the cold room overnight. The insoluble material was filtered off and reextracted with 60 per cent alcohol. The filtrates were combined, distilled under a vacuum to a volume of 100 ml., extracted three times

with ether, and filtered. The final filtrate was concentrated under a vacuum to remove the ether, and the volume adjusted so that a few drops could be used as the daily supplement. All extracts were neutralized with 5 N NaOH before feeding.

Alcoholic extracts of fresh liver, lyophilized liver (Squibb), and defatted whole liver powder (VioBin) produced definite growth responses in rats when fed at daily levels equivalent to 0.11 to 0.45 gm. of dry liver (Table II, Groups 2, 3, 13, 14, 16).

A concentration of the active material contained in these extracts was attempted by adsorption on norit (Darco G-60). 100 ml. of the alcoholic extract were adjusted to pH 2 with concentrated HCl, filtered, and treated with 20 gm. of norit for 1 hour with stirring. The filtrate from the norit was treated with an additional 20 gm. of norit and the residues were combined. Most of the activity was removed, since the supernatant from the adsorption gave little growth, even when fed at 4 times the original level (Table II, Group 4). The elution was carried out by treating the norit with 150 ml. of a 50 per cent methanol solution containing 10 per cent ammonia. Since the first eluate contained little activity (Table II, Group 5), the elution procedure was repeated ten times. The more extensive elution method removed much of the activity from the norit (Table II, Groups 6, 17, 18). The most active preparation which produced a growth response in rats supplied approximately 2 mg. of dry matter per day per rat.

Several commercial liver preparations, which were found to be inactive when fed to rats at lower levels, showed good activity when concentrated by this procedure. Among these preparations was a Lederle liver powder, No. 1432, which was used in subsequent work.

Two groups of rats received an alcoholic extract of fresh liver \cong 0.45 gm. of dried fresh liver per day, but the supplement of one group was heated in a boiling water bath for 15 minutes prior to feeding. Both extracts were equally active in stimulating growth at the levels fed (Table II, Groups 2 and 7).

In order to determine the minimum amount of alcoholic liver extract necessary to give growth stimulation in rats on the mixed diet, the original preparation was diluted 1:2 and 1:4. 3 drops daily of an extract, 1 ml. of which corresponded to 5 gm. of fresh liver, gave a maximum response, while a preparation equivalent to 2.5 gm. of liver per ml. produced nearly maximum response (Groups 13 and 14). The amount of this extract fed daily corresponded to 0.11 gm. of dry liver. Some of the activity remained in the residue after the alcoholic extraction, as shown in Group 15, Table II.

Bosshardt and associates (2) described experiments in which they observed improved growth and utilization of food protein in rats on a casein diet supplemented with 1:20 liver powder or a butanol extract of this pow-

TABLE II

Growth Response in Rats Fed Different Liver Preparations and Other Materials

Series No.	Group No.	No. of animals	Supplement	Level	Average gain per wk. (5 wks.)
					gm.
I	1	6	None		24
	2	4	Alcoholic extract of fresh liver	≈ 0.45 gm. dry liver per day	32
	3	6	Alcoholic extract of lyophilized liver (Squibb)	≈ 0.3 gm. dry liver per day	33
	4	6	Supernatant from norit adsorption of lyophilized liver	≈ 1.2 gm. dry liver per day	28
	5	4	First norit eluate of lyophilized liver	≈ 0.3 gm. dry liver per day	25
	6	6	Norit eluate from lyophilized liver	≈ 0.3 gm. dry liver per day	30
	7	4	Alcoholic extract of fresh liver boiled for 15 minutes	≈ 0.45 gm. dry liver per day	33
	8	4	Butanol extract of Lederle No. 1432 liver preparation	≈ 1.2 gm. dry liver per day	26
	9	4	Sharp and Dohme liver extract, No. 2505	≈ 3 drops per day	33
	10	4	Sharp and Dohme liver extract, No. 2505 injected	≈ 0.35 ml. per wk.	33
II	12	4	None		28
	13	4	Alcoholic extract of fresh liver	≈ 0.22 gm. dry liver per day	35
	14	4	Alcoholic extract of fresh liver	≈ 0.11 gm. dry liver per day	34
	15	4	Residue from alcoholic extract of fresh liver	≈ 3%	33
	16	4	Alcoholic extract of defatted whole liver powder (VioBin)	≈ 0.3 gm. dry liver per day	34
	17	4	Norit eluate of fresh liver	≈ 0.45 gm. dry liver per day	32
	18	4	“ “ “ “ defatted whole liver powder (VioBin)	≈ 0.3 gm. dry liver per day	34
	19	4	Butanol extract from alcoholic extract of fresh liver	≈ 0.45 gm. dry liver per day	28
	20	4	Residue from butanol extract	≈ 0.45 gm. dry liver per day	36
	21	4	Reticulogen, No. 360 (Lilly)	≈ 3 drops daily	36
	22	4	Fish press water	≈ 0.3 gm. dry material per day	33
	23	4	Norit eluate from alcoholic extract of fresh liver stored 6 wks.*	≈ 0.9 gm. dry liver per day	28

* This solution had been highly active at the time of its preparation.

der. We prepared a butanol extract of a powdered liver preparation (Lederle, No. 1432) which had been shown to be active in stimulating growth when concentrated by norit adsorption. This butanol extract was inactive in our assay when fed at a level corresponding to the amount of liver which had been used for the preparation of the active norit eluate (Table II, Group 8). In another experiment our alcoholic extract of fresh liver was extracted seven times with butanol, the butanol was evaporated, and the residue dissolved in water and freed from traces of butanol. This extract was brought to the same concentration as the residue solution with respect to fresh liver, and both solutions were fed to rats at a level of 0.45 gm. of dry weight of fresh liver per day. Only the residue solution showed growth-stimulating activity (Table II, Groups 19 and 20).

Two commercial anti-pernicious anemia liver preparations were tested; Sharp and Dohme, No. 2505 (15 U. S. P. units per ml.) and Lilly reticulogen, No. 360 (20 U. S. P. units per ml.). Both preparations showed full activity when given at a level of 3 drops daily. When given parenterally three times weekly, the Sharp and Dohme extract was equally active in the amount of 0.35 ml. per week (Table II, Groups 9, 10, 21). The possible relation of the unidentified factor stimulating rat growth and the pernicious anemia factor will be tested further.

Finally, a group of rats received the basal diet supplemented with fish press water, since this is known to cause stimulated growth in chicks when fed in addition to a soy bean-corn ration (7, 8). 0.3 gm. of fish press water was fed daily by dropper and a growth effect similar to that caused by liver supplements was observed (Table II, Group 22).

DISCUSSION

The experiments reported in the present paper confirm our earlier finding that the growth of rats fed a diet consisting mainly of whole yellow corn and soy bean meal and supplemented with all the known vitamins is increased when an extract of fresh liver is given. The growth difference between the controls and the liver-treated animals averaged 4 gm. per week on the purified ration and 7 to 10 gm. per week when the mixed ration was used. Nevertheless, there was no significant difference in the growth of the rats in the control groups. This differential effect may be due to the presence of small amounts of growth factors in the "vitamin-free" casein or to variations in the direct and indirect requirement of the animals fed the two diets. Spitzer and Phillips (9) have emphasized the difference between purified and soy bean rations for growth, reproduction, and lactation. Similar differences have been observed in the case of growing chicks (Patton *et al.* (10), and unpublished data).

The active factor in the alcoholic extract of liver is readily adsorbed on

norit, but the elution is difficult and probably incomplete. The first eluate from the norit contained much of the total dry matter adsorbed, but showed very little biological activity, whereas the subsequent treatments removed approximately 50 per cent of the activity from the norit.

It was found that the liver extract retained its activity when heated in a boiling water bath for 10 minutes. However, it must be pointed out that in this experiment an excess of the active factor was fed, since it was later established that, when an alcoholic extract equivalent to 0.11 gm. of dried fresh liver was fed daily to rats on the corn-soy bean diet, more than half maximum growth resulted under the conditions of the experiment. Spontaneous inactivation of some of the alcoholic liver extracts (Group 23), norit eluates, and fresh liver was observed in several instances during storage in the dark and at freezing temperature for relatively short periods of time.

Since no activity was detectable in the butanol-soluble fraction of liver preparations while the extracted residue retained activity, the active factor is probably different from that of Bosshardt *et al.* The fact that commercial concentrated anti-pernicious anemia liver preparations showed activity in rat growth warrants further investigations. The absence of growth-stimulating activity in the butanol extract is of significance in this connection, since Cohn, McMeekin, and Minot reported the anti-pernicious principle soluble in this solvent (11).

Fish press water, which stimulates growth of chicks when fed a natural ration (7, 8), was active when tested with rats. The possibility that this material contains a factor or factors similar to the active principle in liver will be tested in further experiments.

SUMMARY

The growth-stimulating effect of liver extracts has been studied in rats fed a synthetic and a corn-soy bean meal ration containing ten B vitamins and cystine. The effect of liver was more pronounced on the latter diet.

It has been shown that the active material can be adsorbed on and eluted from norit, and that it is soluble in ethanol but not in butanol. Commercial anti-pernicious anemia liver preparations showed considerable activity. A spontaneous inactivation of liver extracts was observed after storage in the cold. Activity was retained after short periods of heating. Fish press water showed a growth-stimulating activity in rats similar to that of liver.

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THE ELEMENTAL AND AMINO ACID COMPOSITION OF CRYSTALLINE CLOSTRIDIUM BOTULINUM TYPE A TOXIN

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The isolation of *Clostridium botulinum* type A toxin in crystalline form (1, 2) has led to an interest in its chemical composition. An apparently complete analysis of the pure crystalline toxin is presented in this paper. Preliminary studies have indicated that the toxin is a protein with an isoelectric point of pH 5.6, possessing the properties of a globulin (1-3). It is free from carbohydrate, halogens, iron, manganese, and magnesium. In addition to carbon, hydrogen, oxygen, nitrogen, and sulfur it contains a small amount of phosphorus and a negligible amount of ash. Nineteen amino acids have been found.

The criteria of purity for the crystalline toxin used in this work are as follows: (1) Ultracentrifugation (4) and electrophoretic studies (1, 2) showed the toxin to be a homogeneous substance. (2) Separately prepared batches of toxin produced under similar cultural conditions showed constant values for the fourteen amino acids determined as an aid in studies of the constancy of composition. (3) Serologically, the toxin acts as a single substance in the toxin-antitoxin reaction.

The material used for these studies was prepared by the method of Lamanna and coworkers (1).

EXPERIMENTAL

Methods

The material used for analysis was dried to constant weight at 100° under high vacuum. In every case, the sample was dried immediately before use. Depending upon the analysis made, the sample was hydrolyzed in (a) 10 per cent HCl, (b) 5 N NaOH, (c) NaOH and SnCl₂, (d) HCl and urea, or (e) HI. The specific directions for the hydrolyses are given in the methods referred to in the literature.

The chemical procedures used and the organism or organisms employed for the microbiological assays are indicated in Table I, together with bibliographic references to the methods. In the determinations by the microbiological methods, five different levels of each amino acid, representing a 5-fold range in concentration, were subjected to analysis. Each

of the five levels was run in duplicate and adjusted so as to fall within the accurate range of the procedure after preliminary assay to determine the approximate amount of amino acid present. Thus ten determinations were made for each amino acid with each organism. Enough toxin was available to carry out an entire analysis on one batch and to determine fourteen amino acids on two other batches. When two different organisms were used and the three batches were subjected to analysis, a total of 60 determinations was made for an individual amino acid. In the determinations by the chemical methods, three different levels in duplicate of the amino acid being tested were subjected to analysis.

Results

The results are given in Table I.

Since similar methods of analysis have been applied to crystalline β -lactoglobulin by Brand *et al.* (23, 24), their general approach to the problem and method of presentation of the results have been followed. Similar assumptions are necessarily made: (1) The toxin is a chemical compound in which the constituent amino acids are present in constant quantities and are joined in the molecule by peptide linkages. (2) The amide nitrogen indicates the amount of glutamine and asparagine present. Since it was not possible to distinguish between these two amides, and since the equivalents of amide nitrogen equal the equivalents of aspartic acid, all of the amide nitrogen has been arbitrarily assigned to the aspartic acid and reported as asparagine. Consequently, all of the glutamic acid is reported as "free glutamic" acid. (3) In the interpretation of the data on the free amino nitrogen of the intact protein, no splitting with the liberation of reactive amino nitrogen occurs during the determination, all of the ϵ -amino nitrogen of lysine is free and reactive, and all terminal free α -amino nitrogen of the peptide chains is free and reactive.

Data are presented for nineteen amino acids. Hydroxyproline was not detected when the colorimetric test of McFarlane and Guest (25) was applied.

The partition of the nitrogen is given in Table I under Determinations 30, 1a, 2a, 3a, 6a, 34, and 35. This sum of 16.29 per cent agrees with the value for total nitrogen (16.29 per cent) as determined by elemental analysis. The value of 11.96 per cent for peptide nitrogen obtained by subtracting the sum of non- α -nitrogen and free α -amino nitrogen from the total nitrogen agrees well with the value for peptide nitrogen obtained by multiplying the per cent water taken up in hydrolysis by 14/18.016, which is 12.07 per cent. These checks on the nitrogen distribution indicate the apparent completeness of the amino acid analysis.

The sulfur partition is given under Determinations 14a + 15a and 16a

TABLE I
Composition of *Clostridium botulinum* Type A Toxin

Determination No.*	Constituent	Method of determination and bibliographic reference No.	Per cent of constituent	Per cent acid residue	Per cent N calculated†	Per cent S calculated‡
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1	Lysine	<i>L. mesenteroides</i> (5)	7.74	6.78	1.48	
2	Histidine	<i>L. mesenteroides</i> (5), <i>S. faecalis</i> (6)	1.03	0.91	0.28	
3	Arginine	<i>L. casei</i> (7), <i>S. faecalis</i> (6)	4.62	4.14	1.49	
4	Tyrosine	<i>L. casei</i> (7), photometric (8)	13.50	12.18	1.04	
5	Phenylalanine	<i>L. delbrueckii</i> (6), <i>L. casei</i> (7)	1.17	1.04	0.10	
6	Tryptophan	<i>L. casei</i> (7), photometric (8), <i>S. faecalis</i> (6)	1.86	1.69	0.25	
7	Valine	<i>L. casei</i> (7), <i>S. faecalis</i> (6)	5.29	4.45	0.63	
8	Leucine	<i>S. faecalis</i> (6), <i>L. arabinosus</i> (9)	10.30	8.91	1.10	
9	Isoleucine	<i>S. faecalis</i> (6), <i>L. arabinosus</i> (9)	11.94	10.33	1.28	
10	Glutamic acid	<i>L. arabinosus</i> (10)	15.57	13.67	1.48	
11	(Aspartic acid)§	<i>L. delbrueckii</i> (11), <i>L. mesenteroides</i>	(20.26)			
11a = 11 + 30	Asparagine¶		20.10	17.34	4.26	
12	Serine**	<i>L. delbrueckii</i> (11)	4.36	3.60	0.58	
13	Threonine	<i>S. faecalis</i> (6), oxidation (12-15), <i>L. arabinosus</i> (16, 17)	8.49	7.19	0.99	
14	Cysteine	Photometric (18)	0.268	0.23	0.03	0.066
15	Half cystine	" (18)	0.534	0.45	0.06	0.142
16	Methionine	<i>L. arabinosus</i> (16, 17), iodometric (19), <i>S. faecalis</i> (6)	1.06	0.93	0.10	0.228
17	Proline	<i>L. mesenteroides</i>	2.60	2.19	0.32	
18	Glycine	" "	1.38	1.05	0.26	
19	Alanine	<i>S. faecalis</i>	3.92	3.12	0.61	
20	Total		115.73	100.20††	16.34	0.436
21 = 20 (Column 4 - Column 5)	Water taken up during hydrolysis		15.53			

TABLE I—*Concluded*

Determination No.*	Constituent	Method of determination and bibliographic reference No.	Per cent of constituent	Per cent acid residue	Per cent N calculated†	Per cent S calculated‡
(1)	(2)	(3)	(4)	(5)	(6)	(7)
22	Carbon	Pregl (20)	53.73			
23	Hydrogen	" (20)	7.02			
24	Total N††	Kjeldahl, Pregl (20)	16.29			
25	" S	Pregl (20)	0.437			
26	Phosphorus	King (21)	0.052– 0.059			
27	Oxygen	Difference	22.46			
14a + 15a	Cysteine S + cystine S		0.208			
16a	Methionine S		0.228			
28 = 14a + 15a + 16a	Protein S		0.436			
29	Sulfate S		0			
30	Amide N	Diffusion (22)	2.13			
1a	ε-Amino N		0.742			
2a	Imidazole N		0.19			
3a	Guanidino N		1.12			
6a	Indole N		0.12			
31 = 30 + 1a + 2a + 3a + 6a	Non-α-N		4.30			
32 = 24 – 31	α-N		11.99			
33	Free amino N	Van Slyke	0.771			
34 = 33 – 1a	" α-amino N	"	0.029			
35 = 32 – 34	Peptide N		11.96			
21a = 21 × N/H ₂ O	" "		12.07			

* Values derived by calculation are designated by "a" (e.g., histidine is Determination 2; imidazole N is Determination 2a).

† Calculated from the percentages of amino acids found by analysis (Determinations 1 to 19).

‡ Calculated from the percentages of amino acids found by analysis (Determinations 14 to 16).

§ The parentheses indicate that aspartic acid is assumed not to be in the molecule. Asparagine is substituted for aspartic acid.

|| Unpublished work of the authors.

¶ All the amide nitrogen is arbitrarily assigned to asparagine.

** Corrected 10 per cent for serine destruction during hydrolysis.

†† The sum of the average values of the individual amino acid determinations. The over-all average deviation from the average is ± 1.8 per cent, based upon the values of 570 determinations. Therefore, the sum of the amino acid residue percentages may be expressed by the figure 100.24 ± 1.8 per cent.

‡‡ Cu-Hg catalyst.

TABLE II

Apparent Number of Moles of Amino Acid Residues Per Mole of Clostridium botulinum Type A Toxin (Mol. Wt. = 900,000)

Lysine	477	Glutamic acid	953
Histidine	60	Asparagine	1370
Arginine	239	Serine	374
Tyrosine	672	Threonine	642
Phenylalanine	64	Cysteine	20
Tryptophan	82	Half cystine	40
Valine	406	Methionine	64
Leucine	708	Proline	203
Isoleucine	820	Glycine	166
		Alanine	394
Total moles.....			7754

TABLE III

Type and Apparent Number of Side Chain Groups in Clostridium botulinum Type A Toxin (Mol. Wt. = 900,000)

Type of group (Table I, determination No.)	No. of groups	Hydrogen bond-forming groups
Cationic groups		
Guanidino (3)	239	239
Imidazole (2)	60	60
Ammonium (33)	495	495
Total cationic groups	794	794
Anionic groups		
Carboxyl (10 + 34)	971	971
Total ionic groups	1765	1765
Non-ionic polar groups		
Sulfhydryl (14)	20	
Half disulfide (15)	40	
Methionyl (16)	64	
Indole (6)	82	82
Phenolic hydroxyl (4)	672	672
Aliphatic hydroxyl (12 + 13)	1016	1016
Amide (30)	1370	1370
Total non-ionic polar groups	3264	3140
Total polar groups	5029	4905
Non-polar groups		
Hydrogen (18)	166	
Paraffin (7 + 8 + 9 + 19)	2328	
Benzene (5)	64	
Pyrrolidine (17)	203	
Total non-polar groups	2761	

(Table I). Methionine, cysteine, and cystine sulfur (0.436 per cent) account for the total sulfur as determined by elemental analysis. This agreement indicates the accuracy of the values for the sulfur amino acids.

The apparent number of moles of each amino acid residue per mole of toxin (mol. wt. 900,000) is given in Table II, and the type and apparent number of side chain groups in the toxin (mol. wt. 900,000) are given in Table III. These values are calculated from the data in Table I.

Minimum Molecular Weight—Since there must be at least 1 molecule of each constituent amino acid per molecule of toxin, this point is used in the case of cysteine and cystine to calculate the minimum molecular weight of the toxin. From the equation

$$M(\text{min}) = \frac{Ri \times Mi \times 100}{\% i}$$

where $M(\text{min.})$ is the minimum molecular weight of the protein and where Mi , per cent i , and Ri are respectively the molecular weight, the content in per cent, and the number of residues of an individual constituent, i , the minimum molecular weight of the toxin is

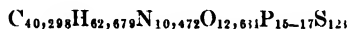
$$M(\text{min}) \text{ cysteine basis} = \frac{1 \times 121 \times 100}{0.268} = 45,149$$

or

$$M(\text{min.}) \text{ cystine basis} = \frac{1 \times 240 \times 100}{0.534} = 44,944$$

These figures are almost exactly one-twentieth the molecular weight of 900,000 as determined by ultracentrifugation and diffusion studies (4).

Suggested Empirical Formulas—If the figure 900,000 is used as the molecular weight of the toxin and the results of the elemental analysis, an empirical formula may be written,



The empirical formula in terms of amino acid residues, according to the suggested convention (24), is as follows:

Gly₁₆₆, Ala₃₉₄, Val₁₀₈, Leu₇₀₈, Ileu₈₂₀, Pro₂₀₃, Phe₆₄, CySH₂₀, (CyS—)₄₀, Met₆₄, Try₈₂, Arg₂₂₉, His₆₀, Lys₄₇₇, (Asp-NH₂)₁₃₇₀, Glu₉₅₇, Ser₃₇₄, Thr₆₄₂, Tyr₆₇₂

DISCUSSION

This work was undertaken with the hope that the results might help to explain the extreme toxicity of this protein. However, no obvious explanation was found. Since the organisms used in the microbiological assays are limited in most cases to the utilization of the natural isomers of

amino acids, it is inferred that the toxin is composed essentially of the L-amino acids connected through peptide linkages. However, the configuration of tryptophan and aspartic acid could not be determined by the methods used; it is possible, therefore, that these two acids may be the D forms. It may also be pointed out that aspartic acid and two hydroxy acids, tyrosine and threonine, are present in somewhat larger amounts than have been found generally in proteins.

In any attempt to explain the toxicity one might consider the following points: (1) the polar nature of the compound; (2) the arrangement of the amino acids within the molecule and the geometrical configuration of the protein as a whole; (3) the possibility of atypical peptide linkages through aspartic acid or glutamic acid, or both; (4) the possible presence of small amounts of a toxic group not detected in the analysis; (5) the large molecular weight.

The phosphorus content of the toxin is subject to some discussion. Phosphorus has been found in all batches with some variation in content. It appears that this element may be either an impurity carried through from the isolation or a slightly variable component of the molecule. However, no detectable absorption was evident at 2580 Å, indicating the absence of nucleic acid as an impurity.

The relatively small amount of toxin available for a complete analysis has necessarily limited the procedures to those in which micro quantities could be used. The results of all microbiological assays and the chemical assays, when they were applicable, showed agreement within 3 per cent.

SUMMARY

The elemental and amino acid composition of crystalline *Clostridium botulinum* type A toxin is presented.

The hydrolysis of 100 mg. of the protein yields 115.73 mg. of amino acids or 100.20 mg. of amino acid residues.

From the data for cysteine and cystine, a minimum molecular weight of approximately 45,000 has been calculated, which is one-twentieth of the molecular weight of 900,000 determined by physical means.

Empirical formulas showing the elemental proportions and amino acid proportions are presented.

No explanation can be given from the analytical data for the extreme toxicity of the protein. The data indicate that the amino acids are biologically active and connected through peptide bonds.

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THE ACTION OF TYROSINASE ON CERTAIN PROTEINS AND PRODUCTS OF THEIR AUTOLYSIS

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The oxidation by tyrosinase of certain proteins, including crystalline preparations of insulin, pepsin, trypsin, and chymotrypsin and partially purified casein, has been reported by Sizer (1). Evidence for protein oxidation was based upon a marked oxygen consumption catalyzed by the enzyme, formation of pigment absorbing strongly in the blue-violet, and a pronounced increase in absorption of the protein in the ultraviolet. In addition, chemical studies on the oxidized protein indicated a decrease in color when tested with Millon's reagent and showed a decrease of 11 to 37 per cent in the tyrosine content. Edman (2), from work with the proteolytic enzymes, decided that Sizer's results may not indicate an effect of tyrosinase upon proteins, but might be explained on the assumption that tyrosinase has acted solely on the small amounts of tyrosine contaminating the proteins or upon the products of autolysis formed during the reaction. His results are based upon the fact that, at the end of the reaction, he could demonstrate appreciable amounts of aromatic amino acids in the filtrate, after precipitation of the digest with trichloroacetic acid. Trypsin was shown to autolyze and, correspondingly, to lose activity. This was not true of chymotrypsin, which was only slightly oxidized by tyrosinase. When pepsin was denatured at pH 7 and then reprecipitated several times at pH 4, Edman could demonstrate little action of tyrosinase on this purified protein. In view of the discrepancies between the results and interpretations of Sizer and Edman, the problem of the action of tyrosinase on certain proteins has been reinvestigated.

Methods and Results

Action of Tyrosinase on Highly Purified Proteins—The technique of the manometric experiments carried out to measure oxygen consumption by purified proteins was identical with that of Sizer (1). Some of the work was done with Upjohn tyrosinase and some with a freshly prepared mushroom tyrosinase (3) containing 17 Adams and Nelson *p*-cresolase units per ml. (4). The substrates were crystalline pepsin, trypsin, chymotrypsin, insulin, and partially purified casein from the same sources as those used previously (1). These preparations before being oxidized were purified in various ways to remove any traces of tyrosine or of

its peptides which might be present. The technique usually employed was to dialyze the proteins (with Cenco cellophane dialyzing tubing) overnight against $m/15$ phosphate buffer, pH 7.3. Typical results on the oxidation of the purified products by tyrosinase are shown in Fig. 1. The

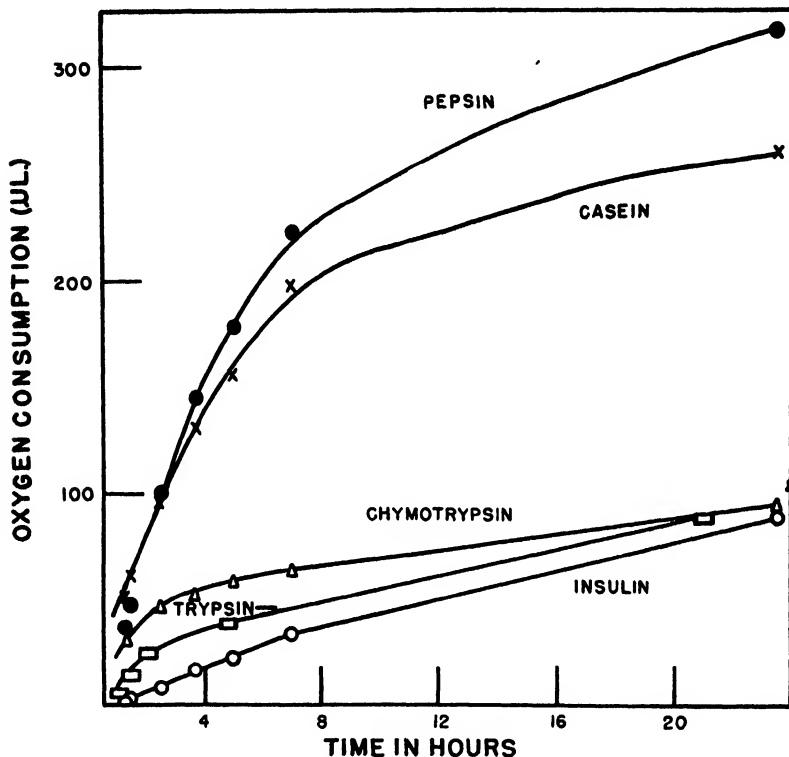


FIG. 1. Oxygen consumption of proteins (purified by dialysis) in the presence of tyrosinase at 37°. The solution contained 25 mg. of protein, 4.5 ml. of $m/15$ phosphate buffer, pH 7.3, 0.5 ml. of Upjohn tyrosinase, and 2 drops of toluene

time curves for oxygen consumption resemble those of Sizer (1) for undialyzed proteins.

For certain of the proteins, other methods of purification or sources of crystalline material were used. Pepsin was purified according to Edman (2) by denaturation at pH 7.0, followed by reprecipitation twice at pH 4.0. A different sample of relatively pure crystalline pepsin¹ was also used. Results were similar to those for the dialyzed protein for both the

¹ Kindly furnished by Professor David Waugh, Massachusetts Institute of Technology.

reprecipitated pepsin (O_2 consumption = 129 microliters in 21 hours) and Waugh's sample of pepsin (O_2 consumption = 319 microliters in 21 hours). Chymotrypsin was purified according to Northrop (5) by salting-out at pH 4.0 with 0.7 saturated $(NH_4)_2SO_4$. Although this sample showed relatively little oxygen consumption resulting from the addition of tyrosinase (22 microliters in 21 hours), the increased absorption in the visible and ultraviolet regions of the spectrum gave evidence that appreciable oxidation had occurred. Recrystallized insulin¹ was oxidized

TABLE I

Effect of Tyrosinase on Oxidation of Purified Proteins and Their Products of Autolysis As Measured by Decrease in Per Cent Transmission of Solution at 410 $m\mu$

Protein	Tyrosinase	Digest before dialysis	Dialyzed protein	Centrifugate*	Super-natant*
Casein	Active	Brown†	Brown	5	32
	Boiled	Gray	Gray	14	57
Chymotrypsin	Active	24	27	20	14
	Boiled	58	44	42	29
Insulin	Active	Brown	Brown	6	79
	Boiled	Gray	Gray	42	83
Pepsin	Active	17	29	8	73
	Boiled	28	49	46	91
Trypsin	Active	25	47		
	Boiled	33	53		

* At the end of the reaction the protein was precipitated from the digest by adding 2 ml. of 10 per cent trichloroacetic acid. The protein centrifugate was redissolved by boiling for 5 minutes in 4.8 ml. of water plus 0.2 ml. of 6 N NaOH.

† When solutions were too turbid to be measured with the Coleman spectrophotometer, only their colors are indicated in the table.

(O_2 consumption = 92 microliters in 21 hours) by tyrosinase about as rapidly as dialyzed crystalline insulin (see Fig. 1).

At the completion of the manometric studies, the solutions were examined for spectroscopic evidence of protein oxidation by tyrosinase. In all cases, such oxidation was indicated by the production of a pigment absorbing in the blue-violet region of the visible spectrum (see Table I) and absorbing strongly throughout the ultraviolet region (see Table II and pepsin curves of Fig. 2). These results are the same as those reported previously (1) where traces of products of autolysis had not been removed from the proteins.

These manometric and spectroscopic experiments clearly indicate that the action of tyrosinase on proteins cannot be accounted for in terms of

an oxidation of products of autolysis, which normally contaminate the proteins.

Does Tyrosinase Act on Proteins Or Only on Their Products of Autolysis Liberated during Reaction?—Although the above experiments clearly in-

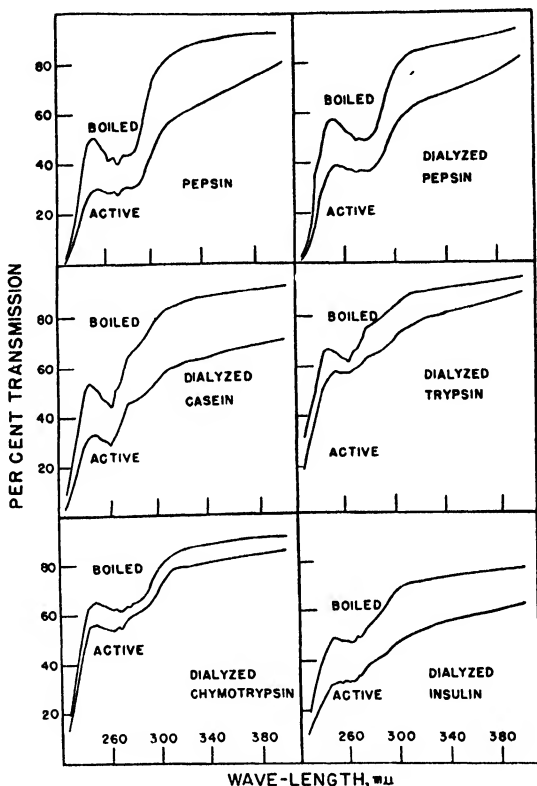


FIG. 2. Absorption in the ultraviolet of proteins (previously purified by dialysis) after oxidation catalyzed by tyrosinase. The control experiments were performed with boiled tyrosinase, while in the companion ones active tyrosinase was used. For pepsin curves, products of protein autolysis were not removed at the end of the reaction; for the other curves the proteins were freed of products of autolysis by dialysis. Solutions were diluted 1:20 with water for spectral analysis.

dicates that the tyrosinase action cannot be attributed to products of autolysis, which contaminate the purified proteins, they do not prove that the effects cannot be explained by the action of tyrosinase on tyrosine or its peptides produced by protein autolysis during the reaction. Under the conditions of the oxidation, pepsin is known to become rapidly denatured and trypsin undergoes considerable autolysis, but no autolysis

of chymotrypsin has been reported (2, 5). Little is known about the possible autolysis of insulin and casein.

After completion of the oxidation of the purified protein by tyrosinase, any products of autolysis were removed by dialysis in the cold room overnight or longer against the phosphate buffer. The residual material was then studied for indications of possible effects of tyrosinase. Results on the pigment formation from proteins, as indicated by absorption in the violet region of the spectrum at $410\text{ m}\mu$ are shown in Table I. It is apparent that roughly similar differences in color between the control (containing boiled tyrosinase) and the experimental solution (containing active tyrosinase) exist in the proteins before dialysis and after the products of autolysis have been removed. The figures, while illustrating clearly the darker color of the solutions treated with active tyrosinase, are only approximate, since occasional slight turbidity of some of the solutions made absolute measurement of color intensity impossible. To the eye, the proteins oxidized by tyrosinase appear brown in color both before and after dialysis, while the control solutions are gray. Results in these experiments were the same whether the preparations were originally purified by dialysis, reprecipitation, or recrystallization.

Another method of determining whether tyrosinase acted upon proteins or their products of autolysis was to separate the components at the end of the reaction by the addition of 2 ml. of 10 per cent trichloroacetic acid to the digest. The precipitates were centrifuged and redissolved by adding 4.8 ml. of water and 0.2 ml. of 6 N NaOH and boiling the mixture for about 5 minutes. A comparison of the color of test and control solutions showed differences (Table I) similar to those Sizer had reported for proteins oxidized by tyrosinase (1). The supernatant solutions containing the products of protein autolysis were also examined and were found to display similar differences in color between experiment and control (Table I). These results in part confirm Edman (2), who found evidence for oxidized products of autolysis after tyrosinase action on proteases.

In view of the characteristic effect of tyrosinase in increasing the absorption in the ultraviolet of certain proteins (1), it seemed advisable to determine whether this effect was eliminated by removing products of protein autolysis either initially contaminating the purified proteins or liberated from the proteins by autolysis during the reaction. Typical results with proteins purified by dialysis, reprecipitation, or recrystallization are presented in Table II and in Fig. 2, pepsin curves, which show that removal of traces of impurities in no way interferes with the usual oxidation of proteins by tyrosinase, as is indicated by increased absorption in the ultraviolet.

Removal by dialysis of products liberated by autolysis during the oxidation, however, has an appreciable effect in decreasing absorption in the ultraviolet of the five proteins (Fig. 2). Since the autolysis is independent of the presence of tyrosinase (2), the absorption of the control solution also decreases, owing to removal of products of autolysis, so that the marked difference between control and experiment is still apparent after dialysis. In all cases, the difference between the two curves is not

TABLE II
Absorption at 276 m μ of Purified Proteins after Oxidation with Tyrosinase

Protein*	Tyrosinase	Per cent transmission	
		Digest	Protein ppt. from digest†
Casein	Active	33	37
	Boiled	46	53
Chymotrypsin	Active	31	58
	Boiled	44	61
Insulin	Active	11	29
	Boiled	37	45
Pepsin	Active	30	36
	Boiled	43	54
Trypsin	Active	39	
	Boiled	56	

* Proteins were purified by dialysis before the addition of tyrosinase.

† Protein precipitated from digest by adding 2 ml. of 10 per cent trichloroacetic acid. The protein was redissolved by boiling for about 5 minutes in 4.8 ml. of water plus 0.2 ml. of 6 N NaOH. All solutions were diluted 1:20 for analysis from 220 to 400 m μ with the Beckman spectrophotometer. Data in the table are only for 276 m μ , at which tyrosine absorbs maximally.

quite as great after dialysis, indicating that a part of the original difference must have been due to the presence of products of protein autolysis oxidized by tyrosinase.

Proteins were also separated by trichloroacetic acid precipitation from the products of autolysis. The redissolved precipitates of oxidized proteins showed differences in absorption in the ultraviolet similar to those shown by the oxidized proteins separated from low molecular weight compounds by dialysis (see Table II). These experiments clearly demonstrate that tyrosinase has produced an oxidation of the protein molecule independent of any additional effect on products liberated during autolysis of a fraction of the protein molecules.

The supernatant trichloroacetic acid solutions were also studied in the ultraviolet. The absorption curves (Fig. 3) indicate the presence of an appreciable amount of low molecular weight derivatives of proteins, and

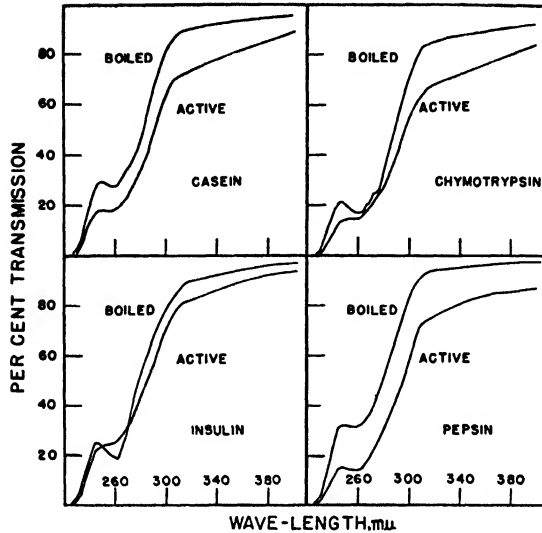


FIG. 3. Absorption in the ultraviolet of products produced by protein autolysis. Control solutions had been treated with boiled tyrosinase, while the experimental ones had been incubated with active enzyme. At the end of the reaction, products of autolysis were separated from the protein by precipitating the latter with trichloroacetic acid. Solutions were diluted 1:20 for spectral analysis.

TABLE III

*Oxidation of Proteins and Their Products of Autolysis by Tyrosinase As Indicated by Changes in Amount of Residual Tyrosine**

Protein†	Tyrosinase	Dialyzed protein‡	Centrifugate§	Supernatant§
		mg	mg.	mg
Casein	Active	0.32	0.36	0.09
	Boiled	0.53	0.39	0.47
Chymotrypsin	Active	0.090	0.11	0.031
	Boiled	0.116	0.15	0.37
Insulin	Active	0.68	0.80	0.031
	Boiled	0.86	0.90	0.086
Pepsin	Active	0.23	0.38	0.060
	Boiled	0.40	0.51	0.343
Trypsin	Active	0.080		
	Boiled	0.113		

* Bernhart's modification of the Millon-Weiss method for tyrosine (6) was used. Each figure represents the average of two to four different experiments.

† Purified by dialysis before addition of tyrosinase.

‡ Dialyzed free of products of autolysis after the oxidation was completed.

§ 2 ml. of 10 per cent trichloroacetic acid was added after the oxidation was completed, and the protein was separated from the products of autolysis by centrifugation.

also show that tyrosinase has produced an increase in the absorption of these products of autolysis. It is not known whether these protein fragments were oxidized by tyrosinase before or after cleavage from the protein molecule, although the latter explanation seems more plausible.

Tyrosine Analyses—Analyses for tyrosine, with Bernhart's modification of the Millon-Weiss method (6), were performed on the purified proteins oxidized by tyrosinase after products of autolysis had been removed either by dialysis or trichloroacetic acid precipitation. In addition, tyrosine analyses were performed on the products of autolysis in the trichloroacetic acid supernatant solutions. Typical results are presented in Table III. Tyrosinase has clearly caused the oxidation of an appreciable fraction of the tyrosine of these proteins, as is indicated by a decrease in the tyrosine of the experimental as compared with the control solution. This is also true of the products of autolysis, since tyrosinase has caused an even greater decrease in the amount of tyrosine which can be measured by the Millon-Weiss method.

DISCUSSION

The results presented in this paper are in general agreement with Sizer's (1) conclusions that certain tyrosyl groups of casein, insulin, pepsin, trypsin, and chymotrypsin can be oxidized by tyrosinase, and cannot be explained by Edman's (2) hypothesis that the enzyme reacts only with tyrosine or its peptides present at the start or liberated by protein autolysis during the course of the experiment.

No new data are presented in this paper on the effect of tyrosinase upon protease activity, since both Sizer and Edman agree that no change is produced. The results do not prove unequivocally that an enzyme with partially modified tyrosyl groups is completely active, since interpretation is complicated by pepsin denaturation and autolysis of trypsin and chymotrypsin, which occur spontaneously under the conditions of the experiment. An approach to this problem might be sought by carrying out the reaction at low temperatures, since protein autolysis has a much greater temperature coefficient than has oxidation catalyzed by tyrosinase (7). In two experiments which were carried out at 25°, the autolysis of all five proteins was much less than at 37°, while the action of tyrosinase was almost as extensive as before.

It is a pleasure to acknowledge the technical assistance of Miss Janette Robinson in this work.

SUMMARY

Removal of traces of tyrosine and its peptides by purification of crystalline pepsin, trypsin, chymotrypsin, insulin, and impure casein did not

interfere with the ability of tyrosinase to oxidize certain of the tyrosyl groups in these proteins. Tyrosinase was shown to oxidize products of protein autolysis, which were liberated during the reaction. Oxidation of these products accounts for only a fraction of the total action of tyrosinase, since the residual protein was also found to be extensively oxidized. Certain of the tyrosyl groups of all of these proteins were demonstrated, by chemical and spectroscopic studies, to have been oxidized by the tyrosinase.

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THE INACTIVATION OF INSULIN SOLUTIONS

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A faintly acid aqueous solution of insulin slowly loses its activity, even at room temperature. There are a number of scattered observations in the literature on the stability of such solutions but Krogh and Hemmingsen (1) were the first to study the phenomenon systematically. They found an optimal stability at pH 2 to 4 and considered the inactivation as a reaction of the first order. According to Sahyun, Goodell, and Nixon (2), addition of zinc salts would have a stabilizing influence, a result we could not confirm (unpublished).

According to our experience, the stability of insulin solutions may vary in quite unpredictable ways and it appeared of some value to reinvestigate the subject, in order to find the causes of the inactivation. Two explanations suggest themselves: hydrolysis or heat precipitation.

The assumption of a hydrolysis as the cause of inactivation gives rise to many difficulties. Jensen and Evans (3) observed that insulin in acid solution loses some ammonia but there is no relation between the loss of ammonia and the rate of the inactivation. Another argument against this hypothesis might be found in the observations of Greenberg *et al.* (4), that several proteins hydrolyze at a rate dependent on the activity of the hydrogen ions. If this relationship should also hold for the assumed hydrolysis of insulin at very mild acidities, it is not easy to explain its optimal stability at pH 2 to 4.

On the other hand there are no objections to the assumption that the inactivation is a denaturation or heat precipitation.

In the course of this work, many experiments were performed in order to find a link between inactivation and hydrolysis. The rate of liberation of ammonia and amino acids has been determined over a range of acidities varying from pH 1.3 to 11. The rate of hydrolysis was found to go through a minimum at the isoelectric point of the insulin and, since the stability of the solutions is definitely better at somewhat higher acidities, any relation between stability and rate of hydrolysis becomes highly improbable. These experiments, confirming and completing Jensen and Evans' work, will, therefore, not be recorded in detail, but during the investigations a method permitting the separation of inactivated from active insulin was discovered. With the aid of this procedure evidence has been accumulated indicating that the inactivation virtually is a denaturation, usually followed by an oxidation process.

Material

All insulin samples were crystalline and air-dry and varied in activity from 19 to 24 international units per mg. Most of them were prepared in our own laboratories and standardized according to the technique of de Jongh, Lens, and Spanhoff¹ on 60 to 80 rabbits each. Two samples of crystalline insulin from other manufacturers were also examined. As no essential difference was found between these samples and our own, the origin of the insulin has not been specified in each experiment.

The commercial solutions investigated were all of our own manufacture.

Technique

It was observed that inactivated insulin can be precipitated quite readily with rather small amounts of electrolyte. This led to the development of a method for its estimation in the presence of insulin. We determined the highest concentration of sodium sulfate at which insulin solutions of pH 3 and of the concentrations used (20 to 100 units per ml.) do not yet precipitate. This proved to be 0.03 N. The solubility of insulin in 0.03 N sodium sulfate is slightly dependent on the pH, and in the first experiments we therefore preferred to determine a small range of the solubility-pH curve with the aid of buffer solutions.

The following example of the technique employed will illustrate sufficiently its simplicity and limitations.

Three 5 ml. samples of a solution containing 5 mg. of insulin per ml. were pipetted into centrifuge tubes. 2 ml. of citrate-phosphate buffer, prepared according to McIlvaine, were added, followed by 3 ml. of 0.1 N sodium sulfate. The tubes were stoppered and mixed by inverting several times. They were left for at least 15 hours at room temperature and then centrifuged. The nitrogen content in the supernatant was determined by the Kjeldahl method.

The same series is made up after the insulin solution, pH 2.95, has been heated for the desired time. Fig. 1 illustrates the result of such an experiment. Curve A, obtained with the unheated solution, shows that the pH during precipitation should be kept between 2.95 and 3.25; otherwise some insulin will also precipitate. On heating, an increasing part of the insulin becomes insoluble in 0.03 N sulfate (Curves B and C).

For routine determinations, we precipitated the inactivated insulin only at pH 3.0.² A determination in the unheated solutions is always necessary, as many of the insulin samples investigated contained small quantities of impurities insoluble in 0.03 N sodium sulfate. As the precipitate formed

¹ de Jongh, S. E., Lens, J., and Spanhoff, R. W., in press.

² Composition of buffer: 8 volumes of 0.1 M citric acid, 2 volumes of 0.2 M disodium phosphate.

is in many instances very strongly hydrated, simple examination of the tubes for cloudiness may be misleading. On the other hand, in some instances the precipitate formed may be quite compact and solid.

The inactivated insulin is very insoluble in the medium described. An insulin solution, containing 5 mg. (100 units) of insulin per ml. was heated for 240 hours at 55°. After the addition of the buffer, pH 3.0, it was precipitated with sodium sulfate; the supernatant contained 0.048 mg. of N per ml. If 4 ml. were first diluted to 10 ml. and then precipitated in the same way, the supernatant contained 0.020 mg. of N per ml. This value is the

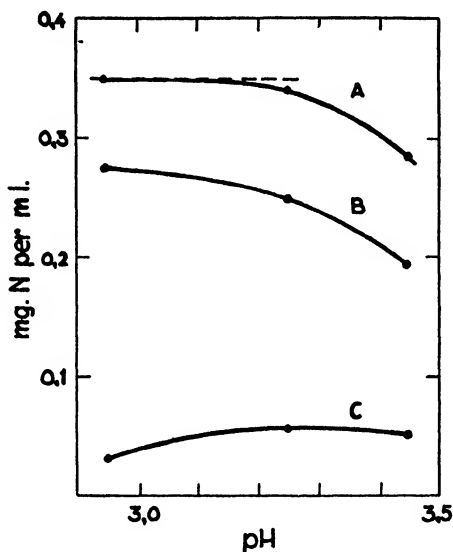


FIG. 1. Influence of time of heating on the solubility of insulin in 0.03 N sodium sulfate. Curve A, unheated solution; Curve B, the same solution heated for 4 hours at 83°; Curve C, solution heated for 18 hours at 83°.

sum of the solubility of the inactivated insulin and of the amount of the original insulin left. If the third point of decimals were reliable, the solubility of the inactivated insulin would correspond to 0.0013 mg. of N per ml., but this value is only very approximate. Furthermore it is probable that the solubility of the inactivated insulin will vary somewhat with the amount formed, but the influence on the curves to be described is considered to be of so little consequence that it has been neglected.

In work with large, rather concentrated batches, it has been observed that considerable quantities of insulin may be lost by adsorption on the precipitate.

In a 2 per cent insulin solution the adsorption may amount to 10 per cent

of the insulin present. In 0.5 per cent or weaker solutions we did not observe any activity of the precipitate which could not be accounted for by its solvent content. Phenolic preservatives should not be present in concentrations over 0.1 per cent as they give rise to a very strong adsorption. They can be removed by dialysis preliminary to the determination.

Results

Rate of Reaction—Determinations were carried out in order to measure the rate of reaction with which inactivated insulin is formed in the pH range 2.9 to 3.0. A few runs were made on the aging effect at 83°, but most of the

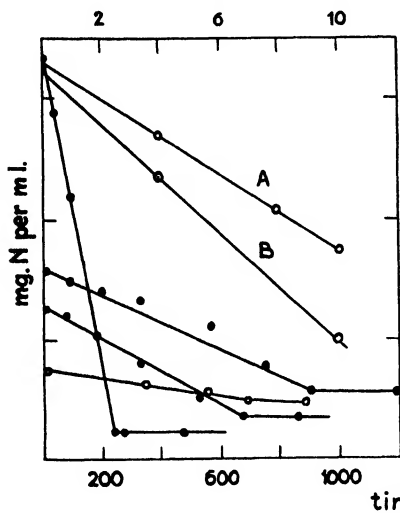


FIG. 2

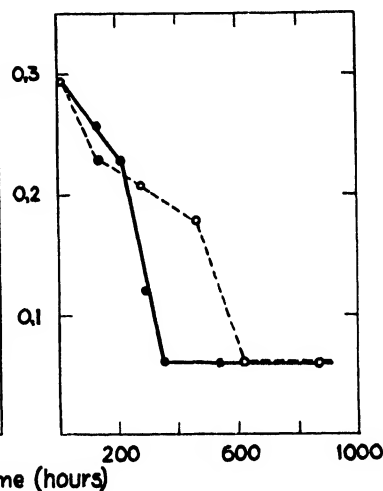


FIG. 3

FIG. 2. Inactivation of insulin solutions, pH 3.0, at 83° (Curves A and B, upper time scale) and at 55° (other curves, lower time scale).

FIG. 3. Inactivation curves of two solutions of the same insulin at 55°.

work was centered on the aging at 55°. In Figs. 2 and 3 some of the curves obtained are represented. Their inspection reveals two unexpected facts. (1) The reaction is apparently of zero order. (2) During the course of the reaction there may or may not be an abrupt change in the rate. The point of the break in a curve is *not* reproducible, as may be seen in Fig. 3 where the aging curves of two insulin solutions of the same sample of insulin, made in the same way, are recorded. The difference is not due to an analytical error and has been confirmed by biological standardization (see below). Fortunately these breaks do not occur often enough to upset every conclusion drawn. Nevertheless the slopes obtained for the "normal" curves are so different for different insulin batches that the influence of

factors affecting the rate of reaction can only be studied by the use of insulin of *one* batch for all experiments.

This has been done in the investigations to be described.

Influence of Surface—No influence of the size of the surface of a solution exposed to glass and to the air could be found. One-half of a solution (50 ml.) was kept in a 300 ml. Pyrex Erlenmeyer flask with an air-exposed surface of 300 sq. cm. and a glass-exposed surface of approximately the same size, the other half being put in a graduate with only 5.5 sq. cm. of air-exposed surface and 85 sq. cm. of glass-exposed surface. Both solutions behaved in the same way as to the formation of inactivated insulin, though

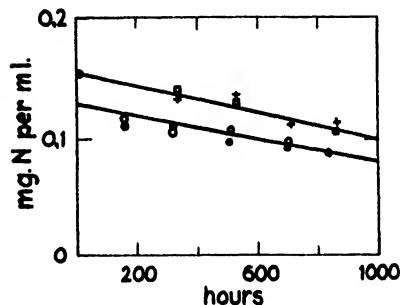


FIG. 4

FIG. 4. The absence of influence on the inactivation of a large surface (\square) and a small surface (+) and of octyl alcohol (\bullet) against a control (\circ). Heated at 55° , pH 3.0.

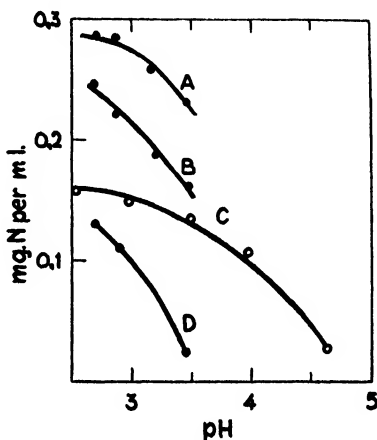


FIG. 5

FIG. 5. The influence of the pH on the rate of reaction. Curve A after 46 hours at 55° , Curve B 94 hours at 55° , Curve D 160 hours at 55° , Curve C 4 hours at 83° .

the solution with the larger surface gradually acquired a golden yellow color. The curves obtained are represented in Fig. 4, together with those on the influence, or the lack of influence, of a highly surface-active substance such as octyl alcohol added in a concentration of 0.1 per cent.

Influence of Acidity—These experiments were performed at 83° and 55° . In order to adjust the solutions of pH 3.2 and higher to the desired pH for precipitation, a buffer of pH 2.85 instead of 3.00 had to be added. A sharp decrease in stability on increasing pH is observed (Fig. 5). Lowering the pH under 2.8 gives rise to the well known heat precipitation in a very short time and to a rapid rise in the ammonium liberation. Hence experiments have not been extended to this pH range.

Correlation between Chemical and Biological Determinations—Confirmation of the chemical results by biological standardization has been obtained in a number of instances. These checks have been extended to a number of determinations of commercial solutions of various ages. In the conversion from chemical to biological values, it has been assumed that 1 i.u. corresponds to 5.4 γ of Kjeldahl nitrogen.

It appears from the curves that the nitrogen in the sulfate solutions does not go down to zero, owing to the presence of impurities which do not share in the process. The calculations are, therefore, based on the decrease in nitrogen rather than on the absolute nitrogen values.

TABLE I
Correlation between Chemical and Biological Determination of Activity of Aged Insulin Solutions

Batch No.	Original activity	Temperature	Time	Activity calculated*	Activity found*
	I. U. per ml.	°C	hrs.	I. U. per ml.	I. U. per ml.
189	40	55	600	14	10
192	89	55	170	63	61
192	89	55	340	51	58
192	89	55	340	4	<10
186	40	55	600	12	10
			mos.		
142b	40	Room	75	22	18 Commercial solution
147m	40	"	70	36	39 " "
175a	40	"	31	37	40 " "
178b	40	"	23	32	28 " "
179b	20	"	23	16	17 " "

* Average difference between observed and calculated values: 0.2 ± 1.3 i.u. per ml. (fourth value not included).

Some results of this comparison between chemical and biological determinations are given in Table I. The assays for this work have been made on ten rabbits in each test. The average difference in calculated and observed activity again shows that the errors introduced by adsorption and incomplete precipitation are either negligible or counter balanced by a possible error in the assumed value of 5.4 γ of N per i.u. of insulin.

The difference recorded in the stability of the commercial solutions is quite striking. Solutions of almost the same age, made and stored in the same way (though from different insulin batches), like Batches 142b and 147m, show a definite difference in stability, which cannot be ascribed to the error of the standardization.

Besides these, the activities of the above solutions, with a large and a small surface, have been compared after 365 and 435 hours, giving equal

values. The same holds for the solutions with and without octyl alcohol, which proved to be equally active after 344 hours.

However, unfortunately there are insulin solutions in which, for unknown reasons, the inactivation takes a different course. Among the scores of solutions investigated there were two, aged at room temperature, which showed no loss of activity by chemical analysis. Yet on biological standardization, less than half the original activity was found.

Properties of Inactivated Insulin—The inactivated insulin precipitated by 0.03 N sodium sulfate does not readily redissolve.

If air has been excluded during the inactivation and the color of the solution has not deepened, regeneration by the well known alkali treatment is, to some extent, possible, but the more the solution has darkened, the less it can be reactivated.

We found that insulin precipitated by heat at pH 2 and in the presence of some electrolyte may be regenerated quite easily and completely, simply by dissolving it in phenol. We used this process repeatedly with large insulin batches and consider it superior to the alkali treatment.

The inactivated insulin dissolves only if it has not become colored in the aging process.

The solutions of inactivated insulin obtained at pH 3 and 55° strongly resemble those of Waugh (5), who subjected insulin to a more drastic treatment. They show a strong double refraction of flow.

A difference in the degree of swelling of inactivated insulin in the pH range 2 to 7 was not observed. In more alkaline solutions the amount of precipitate decreases as the inactivated insulin dissolves.

The inactivated insulin is very stable. The complete transformation from insulin to inactivated insulin is, at 83°, a matter of some 6 to 10 hours, whereas in the following 400 hours at pH 3 and at this temperature no change in the solubility in 0.03 N sulfate can be detected. No amino acids are liberated, for the ultrafiltrate obtained through a soft collodion membrane shows a negative ninhydrin reaction. The Nessler's reaction is positive confirming the well known fact that ammonia is liberated as a secondary reaction.

From an alkaline solution (pH 10.3) no inactivated insulin is formed on aging.

The inactivated insulin usually has a much darker color than the original insulin. The ultra violet absorption is also much greater. The absorption band at 280 m μ has been determined for a number of solutions of different ages, and the results are recorded in Fig. 6. As the acid solutions of the inactivated insulin are somewhat opalescent, the absorption bands have all been measured in a 0.2 M borate buffer, pH 9.3, in which the cloudiness disappears.

The increase in the absorption shows no correlation to the amount of inactivated insulin present, for Curves C and E are those for solutions which have been kept at 55° for 1300 hours, Curve E with a large and Curve C with a small air-exposed surface (see above).

A similar rise in the absorption has been observed by Kuhn, Eyer, and Freudenberg (6) on irradiation of insulin solutions by ultra violet light. Arnow (7) described the effect of the combined action of ultra violet light and oxygen on tyrosine, dopa being the first product formed. The fact that an insulin solution with only a small surface exposed to the air shows much less of the effect than one with a large surface supports the hypotheses that this increase in ultra violet absorption is also due to oxidation, probably

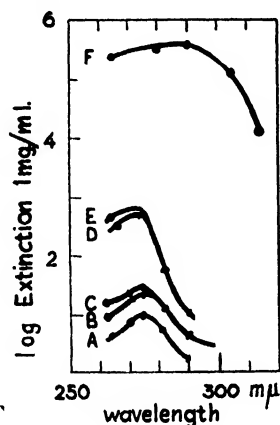


FIG. 6. Absorption spectra (1 mg. per ml.) of a fresh (Curve A) and of aged insulin solutions; Curve B, after 20 days; Curve C, after 56 days (small surface to the air); Curve D, after 58 days; Curve E, after 56 days (large surface to the air); Curve F, after 5 years. All solutions were aged at 55°

of the tyrosine in the molecule. A further indication that oxygen is consumed may be found in the observation that the rubber caps of old commercial vials of insulin frequently show an inward bulge, as if the vial were under a partial vacuum. A direct proof of the oxidation was obtained with a solution of 130 mg. of insulin at pH 3 in a Warburg apparatus which was filled with oxygen. The manometer of the apparatus contained mercury. After 30 days at 55° an oxygen consumption of 0.044 mg. of oxygen per mg. of insulin was observed. This amounts to over 100 gm. atoms per gm. molecule. The dopa reaction of Arnow (8) with nitrous acid and sodium molybdate gives a yellow-brown coloration and, therefore, dopa cannot be present in appreciable amounts. On the other hand, sometimes a strong coloration with dimethylaminobenzaldehyde is observed, indicating the presence of resorcinol-like structures.

DISCUSSION

The predominant cause of the loss of activity of insulin at pH 3 is denaturation. This may be followed by oxidation, which does not influence the first reaction.

Waugh (5), working at a somewhat lower pH, studied apparently the same phenomenon with the aid of the electron microscope. According to his observations no heat precipitation takes place at pH 3, but he heated his solutions for a few minutes only, whereas in these experiments the time of observation extends to many weeks. Waugh was able to show that heat precipitation involves three reactions: formation of active centers, elongation of these centers to fibrils, and finally radial arrangement of the fibrils to spherulites, the visible precipitate. In our experiments the reaction is still more complicated by oxidation and a slight loss of ammonia.

It is doubtful whether the over-all reaction which is measured in these experiments actually is of zero order. Among the simple, irreversible reaction types, the zero order reaction gives the best fitting curve, but such a simple picture of the reaction mechanism is not in accordance with the results obtained with the electron microscope. Therefore the apparent linear decrease in activity can only serve as a first approximation.

The disagreement as regards the order of reaction existing between our investigations and those of Krogh and Hemmingsen is more a matter of interpretation than of actual differences in observations. Their number of determinations is too small and over too small a range to permit the estimation of the order of the reaction.

Our previous experience about the unpredictable extent of the inactivation has been confirmed. The stability of insulin is only a fictitious figure if it is given without the restriction for one particular solution of one particular batch. That is the reason why no great significance is attached to the fact that the results of the stabilizing influence of zinc, observed by Sahyun, Goodell, and Nixon, could not be confirmed in our experiments, checked by biological test, before this investigation had begun.

The method offers a means of checking the purity of insulin batches in which inactivated insulin is present as a contamination of the crystals. The zero value of the aging test can supply this information.

SUMMARY

1. A method is described for the determination of acid-inactivated insulin by making use of its low solubility in 0.03 N sodium sulfate at pH 3.0.
2. This method has been used for the determination of the stability of insulin solutions. The rate of reaction is quite unpredictable and may show a sudden change while the reaction is proceeding, but it is, within a

series, dependent on temperature and pH and not on the size and condition of the outer surface. It is essentially a denaturation reaction.

3. The denaturation may be followed by an oxidation of the denatured product, making the process irreversible.

4. The inactive insulin does not easily hydrolyze.

5. In some exceptional cases, for unknown reasons, the inactivation takes a different course without the formation of the above product.

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OXIDATIVE DECOMPOSITION OF VITAMIN A

I. STABILITY OF VITAMIN A TOWARDS OXIDATION AND IRRADIATION

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In recent years a considerable amount of attention has been centered about the stability of vitamin A towards the effects of solvents, irradiation, and oxidation. Many statements made in this connection are contradictory to the findings of some workers. Such apparent discrepancies may be reconciled, however, when the source of the vitamin in terms of natural protective substances as well as the purpose and method of the experiment is considered.

The present investigation is designed to throw further light on the stability of vitamin A towards oxidation and irradiation. The nature of the solvent and the concentration of the vitamin are taken into account in order to correlate the data on a more concrete basis than has been published hitherto.

EXPERIMENTAL

Equipment and Reagents—The polarograph, employed to determine the oxidative strength of the solvent by the ferrous-ferric ion method, was constructed according to the direction of Kolthoff and Lingane (8). The instrument was equipped with a compensating circuit so that galvanometer readings would be directly proportional to the ferric ion concentration.

A Beckman quartz prism spectrophotometer, model D360, equipped with the ultraviolet attachments, was employed to determine the concentration of vitamin A at 328 m μ . We can be certain that carotenoids other than vitamin A are absent from soup-fin shark liver oils. Unsaturated glycerides and other substances do not markedly affect the shape of the absorption curves of high potency oils (2). If low potency oils had been used in these experiments, correction factors would have been necessary to give values truly representative of the vitamin A content of the oils.

Soup-fin shark livers (100,000 to 500,000 i.u. per gm. of oil) obtained from the California Division of Fish and Game, Bureau of Commercial

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Fisheries, were frozen in dry ice at the time of catching and kept in this condition until ready for use. The livers were then ground, while frozen, in a meat chopper and homogenized in a blender under a stream of nitrogen. An excess of sodium chloride was added to salt-out the tissue. After storing at 10° for 2 days under nitrogen, the clear supernatant oils were decanted and used as such.

Crystalline vitamin A acetate (Distillation Products, Inc.) was dissolved in triacetin (Eastman Kodak Company) to give a 0.5 per cent solution by weight.

Mixed tocopherols (40 per cent) (Distillation Products, Inc.) were employed as the antioxidant.

Peroxide-free diethyl ether was prepared by shaking c.p. diethyl ether with a 20 per cent solution of sodium bisulfite, then with dilute sodium hydroxide, and finally with water until the washings were alkali-free. The ether was then tested with potassium iodide and sulfuric acid to ascertain the absence of peroxides. In certain cases the ether was purified by shaking it with 5 per cent ferrous ammonium sulfate dissolved in 3 per cent hydrochloric acid and an equal volume of concentrated hydrochloric acid, then with water until the washings were free of acid.

Decomposition under Forced Aeration—Various soup-fin shark liver oils as well as 0.5 per cent crystalline vitamin A acetate dissolved in triacetin were employed to study the effect of forced aeration at temperatures ranging between 70–100°.

About 20 ml. of oil were placed in a Pyrex test-tube provided with a two-hole stopper. A capillary whip, inserted into one of the holes, extended to the bottom of the test-tube, while the other hole was connected to the suction pump. The test-tube was then immersed in a constant temperature water bath and air was aspirated through the oil at a rate of about 2 liters per minute. Aliquots were drawn off from time to time, weighed, and diluted with isopropanol. The optical density of the resulting solutions was measured at 328 m μ .

The time required to decompose 50 per cent of the vitamin was arbitrarily taken as a measure of the stability of the vitamin in the oil under the particular conditions of the experiment. Typical decomposition curves have been presented in previous papers (1, 2). As a result of a similar study on three different oils decomposed at various temperatures, the temperature coefficient of the reaction was calculated. The average value for every 10° rise in temperature in the range studied was 2 and varies from 1.6 to 2.2. Wokes and Willimott (12), working with cod liver oil, found a temperature coefficient of 1.4 to 1.5 per 10° rise in temperature.

Aeration of soup-fin shark liver oils resulted in a long induction period followed by a rapid oxidation of vitamin A, in agreement with the findings

of Chevalier *et al.* on vitamin A esters (3). The time of the induction period decreased as the temperature of the aeration increased.

Stability values obtained on various soup-fin shark liver oils varied between 52 and 175 minutes. The variation in the values appears to be characteristic of the particular oil, since all samples had been kept under dry ice until used and no significant oxidation could have taken place prior to the test. This observation suggests that the oils from the livers of individual sharks contain varying amounts of antioxidants. Other investigators (5-7, 9) working with other oils have reported that the addition of mixed tocopherols to an oil increases the stability of the oil

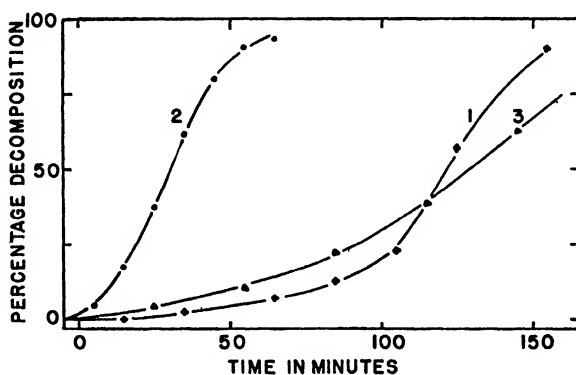


FIG. 1. Influence of mixed tocopherols on the rate of decomposition of a partially oxidized soup-fin shark liver oil. Curve 1 was obtained on fresh oil; Curve 2 was obtained after the oil had been allowed to stand 2 months at room temperature; Curve 3 demonstrates the regenerative effect of tocopherols on the antioxidant activity of the oil. The decomposition was initiated 1 hour after the addition of tocopherols.

to a marked extent; this is also true in the case of soup-fin shark liver oils (2), but tocopherols do not prevent the ultimate decomposition of the oils. The addition of 0.5 per cent mixed tocopherols to an oil which had been allowed to stand at room temperature in an open bottle restored a large portion of the stability of the vitamin (Fig. 1). Curve 1 was obtained on the fresh oil, Curve 2 was obtained on the same oil after it was allowed to stand 2 months in an open bottle in the laboratory (the sample was shaken daily), and Curve 3 demonstrated the effect obtained when 0.5 gm. of mixed tocopherols was added to 100 gm. of the partially decomposed oil.

The high degree of stability of vitamin A in soup-fin shark liver oils indicated by these experiments is not to be misinterpreted. Even though the oils had been drastically treated and showed no loss of vitamin A in

the initial lag period of the decomposition, the oils had lost the greater part of their antioxidant activity and had become rancid. This has been more clearly shown in Figs. 2 to 5 of previous publications (1, 2). The results showed that, although the potency remained the same over a period of time, the stabilities of the vitamin towards aeration decreased as much as 80 per cent. Sanford and Harrison¹ reported similar results on the stability of vitamin A in dogfish liver oils.

Decomposition of Dilute Solutions—A number of soup-fin shark liver oils and of vitamin A ester concentrates were dissolved in diethyl ether and in methylene chloride. One-half of the resulting solutions were placed in the dark; the other half were kept in diffused light. Aliquots were

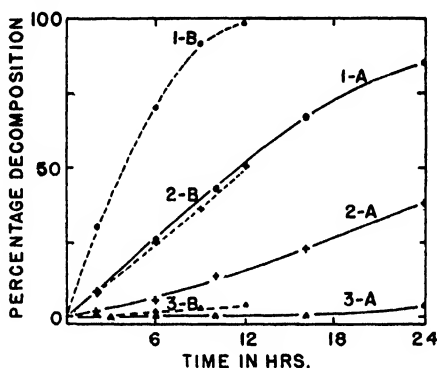


FIG. 2. Stability of a natural vitamin A ester concentrate in refrigeration grade methylene chloride. Effect of oil concentration on the rate of decomposition in the absence of light (A) and in the presence of light (B). Curves 1, 2, and 3 represent, respectively, 0.002, 0.04, and 1.0 per cent oil in methylene chloride.

drawn at various intervals, the solvents removed *in vacuo*, and isopropanol added as required. The optical density at 328 $m\mu$ of the resulting solutions was determined in the Beckman quartz prism spectrophotometer. In all cases the percentage decomposition was greater in the presence of light and in the more dilute solutions (Fig. 2). Thus a 1 per cent solution of a soup-fin shark liver oil in methylene chloride showed only about 4 per cent decomposition in the course of 12 hours when exposed to diffused daylight, while a 0.002 per cent solution of the same oil in methylene chloride was 98 per cent decomposed in the same period.

The addition of mixed tocopherols to fresh dilute solutions (Fig. 3) markedly retarded the rate of decomposition, suggesting that the decomposition is of an oxidative nature. Similar results were obtained with

¹ Sanford, F. B., and Harrison, R. W., Technological Laboratory, United States Department of the Interior, Seattle, Washington; personal communication.

diethyl ether as the solvent (1). That the decomposition is the result of oxidation is further borne out in the following experiment. Treatment of the above solvents with ferrous ammonium sulfate resulted in a rapid oxidation of ferrous to ferric ion, as determined polarographically. The solvents thus reduced were not as effective as oxidants for ferrous ion (2) and for vitamin A (Fig. 4) as were the untreated solvents. That the solvents retained a fraction of their oxidizing power was undoubtedly the result of dissolved oxygen.

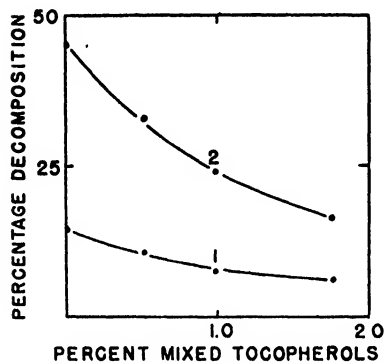


FIG. 3

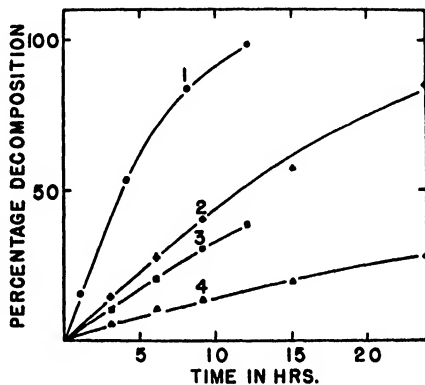


FIG. 4

FIG. 3. Effect of mixed tocopherols on the irradiative decomposition of a soup-fin shark liver oil dissolved in refrigeration grade methylene chloride. The time of irradiation was 4 hours for Curve 1 and 22 hours for Curve 2. The oil concentration was 0.03 per cent.

FIG. 4. Effect of purification of methylene chloride on the rate of decomposition of dilute vitamin A solutions. Curves 1 and 3 were obtained on solutions kept in the presence of light, while Curves 2 and 4 were obtained on solutions kept in the dark. The solvent employed for Curves 1 and 2 was untreated, while that used for Curves 3 and 4 had been previously treated with ferrous ion in the presence of dilute hydrochloric acid.

The dilute vitamin A solutions contained the equivalent of 3 to 4 γ of vitamin A per ml. The solubility of oxygen in the solvents is undoubtedly great enough to oxidize vitamin A to a noticeable extent in such dilute solutions.

DISCUSSION

Evers (4) states that vitamin A in cod liver oil is destroyed by light and oxidation and that the chief cause of the decreased potency of such oils is light. He remarks also that the addition of 0.05 per cent hydroquinone does not prevent the destructive effect of light. It is shown here that heat and light catalyze the oxidative decomposition of vitamin A

and that mixed tocopherols have good antioxidant properties. In this connection we must remember that antioxidants do not prevent the ultimate oxidative destruction of a compound, but merely retard the rate of the decomposition.

Smith *et al.* (10) irradiated alcoholic solutions of vitamin A with ultra-violet light of a wave-length exceeding $300\text{ m}\mu$. According to these authors this treatment resulted in a progressive decrease in the E value at $328\text{ m}\mu$. The more intense the irradiation, the more rapid was the destruction of the $328\text{ m}\mu$ band. When these irradiated solutions were allowed to stand in the dark, the E value at $328\text{ m}\mu$ increased towards the original value. The greater the "decomposition," the less complete was the recovery, which indicates, according to these authors, that vitamin A undergoes a reversible photochemical isomerism accompanied by an irreversible destruction. We did not notice any restoration of the E value at $328\text{ m}\mu$ in our samples subjected to this treatment. Instead we found a further though less marked decrease in the E value at $328\text{ m}\mu$ which we attributed to dissolved oxygen. Supplee and Odessa (11) found that irradiation of milk, either dry or liquid, did not result in the destruction of vitamin A or in the production of toxic symptoms when such milk was fed to rats.

From the above it is quite certain that in any statement regarding the stability of vitamin A the ratio of the vitamin to dissolved oxygen or peroxides and the presence and nature of antioxidants must be taken into consideration. Light and heat catalyze the oxidative decomposition of vitamin A, the former probably through the intermediate formation of an activated vitamin A molecule.

SUMMARY

Various soup-fin shark liver oils and vitamin A preparations have been subjected to forced aeration at various temperatures. The stability of vitamin A, defined as the time required to decompose 50 per cent of the vitamin under aeration at 100° , varies between 52 and 175 minutes for soup-fin shark liver oils. The temperature coefficient of the reaction was found to be close to 2 for every 10° rise in the temperature range from 70 – 100° . The length of the induction period varied inversely with the temperature of aeration.

Dilute solutions of vitamin A on standing at room temperature show a greater percentage decomposition than do the more concentrated solutions. The decomposition seems to be an oxidation that is catalyzed by light and heat.

The research was carried out under the general direction of Dr. J. Murray Luck to whom I extend my grateful appreciation for his keen interest

in the work and for his valuable criticisms and suggestions. I am also indebted to the Division of Fish and Game, Bureau of Natural Resources, State of California, for the generous funds which made this work possible.

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OXIDATIVE DECOMPOSITION OF VITAMIN A

II. ABSORPTION SPECTROPHOTOMETRY OF OXIDIZED VITAMIN A

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A number of investigators (2, 4, 5, 9-14) have demonstrated that vitamin A is readily decomposed under the influence of ultraviolet light. The effect of irradiation on the absorption spectrum of vitamin A has been studied by Dubouloz (5), Chevalier (3, 4), and by Sobotka *et al.* (13). These authors found that ultraviolet light induces a series of changes in the shape of the absorption curve, characterized by the appearance of new maxima at the expense of the 328 $m\mu$ chromogen. The concentration of vitamin A in their solutions was of the order of 3 to 4 γ per ml., a concentration that may be employed to develop the spectrogram without resorting to further dilution. Although such low concentrations of the vitamin do not permit isolation of the degradation products, these authors entertained the plausible belief that their solutions contained traces of oxygen which could not be removed by the usual methods and that the molecular ratio of oxygen to vitamin A was sufficiently high to oxidize noticeably the vitamin under the influence of ultraviolet light. Thus the appearance of new maxima of wave-lengths shorter than 328 $m\mu$ would be the result of oxidative products, while those appearing at wave-lengths longer than 328 $m\mu$ would be dependent upon polymerization of some degradation products, or possibly be due to the production of axerophthol, the aldehyde corresponding to vitamin A (2-5, 8, 12, 13).

The present investigation has been designed to prove that the changes in the shape of the absorption spectrum of vitamin A under the influence of irradiation are the result of oxidation catalyzed by irradiation.

EXPERIMENTAL

Preparation of Reagents--Soup-fin shark liver oils were prepared as previously described (1).

Crystalline vitamin A acetate (Distillation Products, Inc.) was dissolved in triacetin (Eastman Kodak Company) to give a 0.5 per cent solution by weight.

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Absolute ethyl alcohol was prepared according to the method given in Gilman (6).

Peroxide-free diethyl ether was obtained by shaking c.p. diethyl ether with a 20 per cent solution of sodium bisulfite, then with dilute sodium hydroxide, and finally with water.

Method

Various soup-fin shark liver oils and crystalline vitamin A acetate were aerated at various temperatures in the presence and in the absence of diffused light, as described in a previous paper (1). Weighed aliquots were withdrawn from time to time and diluted with isopropanol. The ultraviolet absorption spectra of the resulting solutions, contained in 1 cm. quartz cells, were obtained with a Beckman D360 quartz prism spectrophotometer equipped with a hydrogen discharge lamp and an ultraviolet-sensitive phototube. The readings were taken at intervals of 2 $m\mu$ in the range of 214 to 400 $m\mu$. Four or five points along the curve were redetermined at the conclusion of a run in order to check the stability of the solutions in the instrument. The check results were in good agreement for the most part, although discrepancies were encountered at times below 220 to 230 $m\mu$.

DISCUSSION

The aeration of soup-fin shark liver oils produces a succession of changes in the shape of the absorption curves of the resulting solution in isopropanol (Fig. 1). As decomposition progressed, the maxima of the successive absorption curves shifted toward lower wave-lengths. These maxima were located at 326 to 328, 310 to 312, 294 to 296, 284 to 286, and at 274 to 275 $m\mu$. Similar results were obtained with vitamin A acetate. In addition to these maxima, soup-fin shark liver oils showed an inflection or maximum at about 230 $m\mu$, while vitamin A acetate showed only an increased absorption in this region, with a possible maximum located at a wave-length lower than 220 $m\mu$. This last band became more pronounced as decomposition progressed. The extinction coefficient at about 290 $m\mu$ was found to be fairly constant for any given oil irrespective of the extent of the aeration. Similar results have recently been published (7).

The effect of the aeration temperature on the resulting absorption spectrum was found to be negligible with the exception that the rate of reaction was faster and that the extinction coefficient of the bands below 295 $m\mu$ tended to be lower as the aeration temperature was raised. This is probably the result of increased volatility of some of the products at the more elevated temperatures. Aeration in the presence of light did not cause the shape of the resulting absorption curves to differ from those

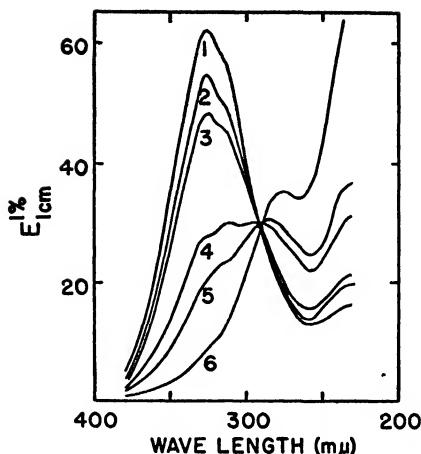


FIG. 1. Effect of aeration on the absorption spectrum of soup-fin shark liver oil. Curves 1 through 6 were obtained respectively at 0, 8, 9, 12½, 15, and 20 hours aeration at 60°.

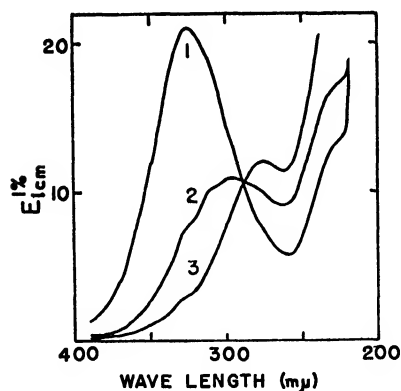


FIG. 2

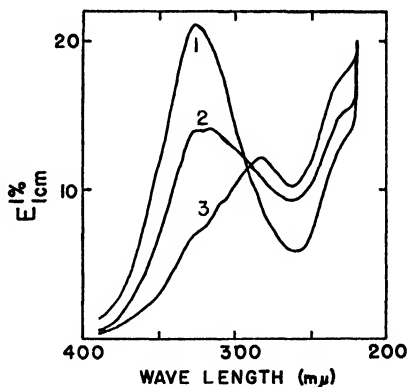


FIG. 3

FIG. 2. Absorption spectra of a 0.002 per cent isopropanol solution of a soup-fin shark liver oil allowed to stand at room temperature in the dark. Curves 1 through 3 represent, respectively, 0, 14, and 38 hours standing.

FIG. 3. Absorption spectra of a 0.002 per cent isopropanol solution of a soup-fin shark liver oil allowed to stand at room temperature in the presence of light. Curves 1 through 3 represent, respectively, 0, 2, and 4 hours standing.

obtained by aeration in the dark. Similar results were obtained on dilute isopropanol solutions of the oils kept standing in the dark (Fig. 2) or in the presence of light (Fig. 3).

It will be recalled from an earlier paper (1) that vitamin A in dilute solutions exhibits decomposition more readily than in more concentrated

solutions, and that the rate of decomposition is also proportional to the "peroxide number" of the solvent employed and the temperature at which the solution is kept. It is quite apparent that each reaction is characteristically the same; namely, an oxidation catalyzed by light and heat.

The product absorbing at 276 to 278 $m\mu$ is probably identical with that resulting from the irradiative decomposition of vitamin A reported by Sobotka *et al.* (13). There is a striking similarity between the shape of the curves presented in this paper and those reported on irradiated vitamin A alcohol (13) and on irradiated halibut liver oils (5), suggesting that irradiation of vitamin A-containing oils produces secondary oxidative reactions. Aeration did not produce the 346 $m\mu$ and the 364 $m\mu$ bands resulting from the irradiation of vitamin A acetate (13) or of halibut liver oils (5).

SUMMARY

Aeration of vitamin A acetate dissolved in triacetin and of soup-fin shark liver oils or long standing of diluted solutions of these oils both in the presence and in the absence of light resulted in the successive appearance of maxima towards lower wave-lengths. These were located at 326 to 328, 310 to 312, 294 to 296, 284 to 286, and 274 to 275 $m\mu$. The shape of the curves was essentially the same as of those reported by Sobotka *et al.* (13) and by Chevalier and Dubouloz (4) on irradiating vitamin A-containing oils. It is, therefore, believed that irradiation produces a secondary photooxidation that is responsible for the changes in the absorption spectrum of vitamin A reported by others.

The research was carried out under the general direction of Dr. J. Murray Luck to whom I extend my grateful appreciation for his keen interest in the work and for his valuable criticisms and suggestions. I am also indebted to the Division of Fish and Game, Bureau of Natural Resources, State of California, for the generous funds which made this work possible.

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A RAPID METHOD FOR THE PRODUCTION AND ISOLATION OF PENICILLINASE

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Production and purification of the extracellular penicillinase from *Bacillus cereus*, NRRL 569, have been reported by LePage, Morgan, and Campbell (3). The procedure employed consisted of adsorption on Hyflo Super-Cel, elution with dilute ammonia water, ammonium sulfate fractionation, dialysis, and lyophilization. By a similar purification method subsequently published by Housewright and Henry (2) partially purified enzyme of relatively low activity was obtained.

The increasing application of penicillinase to testing penicillin sterility has made the development of simpler and more rapid methods of production highly desirable. The present investigation was therefore undertaken.

EXPERIMENTAL

Previous investigations on the production of penicillinase by *Bacillus cereus*, NRRL 569, had shown that addition of excess penicillin early in the growth period caused lysis of a considerable proportion of the cells. Recently, with penicillin of greater purity at the same unitage levels, this observation was no longer noted. It was assumed therefore that at least part of the lytic effect might be due to one or more impurities in the penicillin preparations. At the same time, it was felt that greater advantage could accordingly be taken of the adaptive nature of the enzyme by adding more substrate during the early stages of growth with the possibility of reducing the production time.

A relatively pure penicillin G (1400 units per mg.) and salts with mixed penicillins ranging in purity from 500 to 1000 units per mg. were tested. While in most cases impure material was reasonably satisfactory, more reproducible results were obtained with pure penicillin. The lack of sufficient high purity preparations for these studies would have made the development and use of a synthetic medium meaningless at this time.

It had been pointed out previously (3) that lots of yeast extract varied in their suitability for enzyme production. Certain lots, while supporting a satisfactory growth of *Bacillus cereus*, were not suitable from the standpoint of enzyme yield. Moreover, the pH tended to rise to 8.0, causing difficulties in assays and enzyme recovery. With the incorporation of 0.1 to

0.2 per cent glucose in such media reasonably high titers could be obtained in 24 hours and growth was maintained at neutrality.

In practice, however, it has been found advisable to test small amounts of various batches of yeast extract and select one for use which, in the absence of penicillin, produces a titer of at least 128 dilution units per ml. (3) in 24 hours. Such lots are then used without glucose addition.

Method—With a satisfactory lot of yeast extract and the medium previously reported (3) carboys are seeded with a 2 per cent inoculum of an 18 hour *Bacillus cereus* broth culture. These are maintained at 22–26° with rapid aeration. At the end of 3 hours 1 unit of high potency penicillin is added per ml. of culture medium. At 5, 7, and 9 hours, penicillin additions to final concentrations of 4, 10, and 25 units respectively are made. The penicillin is diluted in small quantities of sterile distilled water prior to addition to the culture.

In 48 hours titers are sufficiently high to warrant recovery of the enzyme. With optimal conditions titers greater than 20,000,000 dilution units per ml. have been reached. Higher yields of enzyme are obtained if greater amounts of penicillin are added but a longer growth period becomes necessary.

At the end of the incubation period the culture is passed through a Sharples supercentrifuge and the cell-free culture filtrate retained for enzyme recovery.

Assay—The method of assay outlined in the previous publication was used (3), a "dilution unit" being defined as that dilution of enzyme in phosphate buffer, pH 7.0, which causes 50 per cent inactivation of 1 unit of penicillin in 1 hour at 37° as tested by a cup-plate assay. With the development of more active filtrates and of highly purified enzyme, end-points have become increasingly difficult to obtain. Complete inactivation of penicillin was frequently found in dilution series carried to thirty tubes. For this reason the tube dilution assay becomes somewhat unsuitable when used to test highly active materials.

It was observed that broths of pH 8.0 could not be assayed accurately without preliminary adjustment to pH 7.0. When this was not done, significantly lower end-points were obtained. This observation reaffirms the sharp pH optimum for penicillin inactivation previously suggested (3).

In spite of these objections this assay provides a reasonably quick and accurate estimation of penicillinase activity. In addition potency of all final enzymes is further assayed by testing the activity of 1 mg. against graded amounts of concentrated penicillin solutions.

Isolation of Enzyme

Precipitation—In an endeavor to find a suitable method to replace the cumbersome adsorption and elution technique, the effect of addition of acid

to the cell-free culture filtrate was studied. Preliminary experiments indicated that adjustment of the culture liquor to pH readings of approximately 4.5 served to precipitate the enzyme, which could then be recovered by filtration through fritted glass filters. Detailed studies were accordingly carried out to determine the optimal conditions for enzyme precipitation.

A 5000 ml. volume of cell-free filtrate was divided into five equal portions. The pH of these lots was adjusted with glacial acetic acid to several points in the acid range. All acid additions were carried out gradually with mechanical mixing at a temperature of 5°. After allowing 4 hours at 5° for floccule formation, the precipitated material was filtered off, redissolved in 0.5 per cent ammonia water (3), and the enzyme reprecipitated by 100

TABLE I

*Relationship of pH to Precipitation of Penicillinase from Culture Filtrates**

The results are expressed as dilution units multiplied by dry weights of material.

pH†	Activity recovered‡
5.8	30,400,000
5.4	56,500,000
5.0	31,600,000
4.6	3,700,000
4.2	80,000

* Original activity of culture filtrate, 65,000,000 dilution units per liter.

† Initial pH of the culture filtrate, 7.2.

‡ All activities reported are based on 1 liter of cell-free culture filtrate.

per cent ammonium sulfate saturation. After dialysis and lyophilization penicillinase activity was measured. Typical results are presented in Table I.

From these results it would appear that adjustment of the pH to 5.4 gave maximal yields of penicillinase, and that pH values much lower than 5.0 resulted in serious loss of enzyme. Subsequent experiments have established that reduction of the culture filtrate to pH 5.2 yielded optimal recoveries.

Further reduction of the pH of all the above fractions to 3.8, after removal of the originally precipitated material, did not lead to further recovery of penicillinase. Enzyme activity appeared to be lost when the culture filtrate was allowed to stand at low pH levels.

Adjustment of the clear culture liquid to pH 5.2 resulted in the appearance of a very fine diffuse turbidity. After standing at 5° for 2 hours, tiny floccules appeared which became larger after 24 hours. To determine whether losses occurred at the flocculation stage the relationship of time to enzyme precipitation was therefore investigated.

A 2000 ml. volume of culture liquid was adjusted to pH 5.2 with glacial acetic acid and immediately divided into eight equal portions. One aliquot was filtered at once, while the remaining seven were filtered after being held at 5° for periods of $\frac{1}{2}$, 1, 2, 4, 8, 12, and 24 hours respectively. Residual activity in the filtrates was then measured. Irrespective of whether filtration was carried out immediately or was deferred for periods up to 24 hours, no significant differences could be detected in amounts of enzyme lost in the filtrates.

Comparison of the activity of the original culture liquor with the activity remaining after acid precipitation showed clearly that very little loss of penicillinase took place at this step. In general, approximately 0.1 per cent of the original activity remained in the filtrates and losses greater than 1.0 per cent were never encountered. These values compare favorably with the 4 per cent loss previously shown to occur during Super-Cel adsorption (3).

Precipitation with acids other than acetic was also tested. No appreciable differences in either yield or activity of final enzyme could be obtained when the culture filtrate was acidified with hydrochloric, sulfuric, phosphoric, or acetic acids.

Attempts to remove precipitated penicillinase from the culture filtrate by repeated passage through a Sharples supercentrifuge resulted in considerable loss of enzyme. It was found that quantitative recovery could be achieved by passage through fritted glass filters, provided an F porosity was used.

Fractionation—Purification of acid-precipitated penicillinase was first attempted by fractionation at 50 and 100 per cent ammonium sulfate saturation. Contrary to results in previous studies (3), variable amounts of enzyme, representing up to 75 per cent of the total activity, were precipitated at 50 per cent saturation. Extensive investigations were accordingly conducted to determine optimal conditions for the fractionation procedure.

A 5000 ml. volume of cell-free culture filtrate was acidified to pH 5.2 and the precipitated enzyme removed by filtration. The precipitate was dissolved from the filters in a total volume of 1000 ml. of 0.5 per cent ammonia water and was then divided into ten 100 ml. portions. To each lot solid ammonium sulfate was added to give increasing degrees of saturation. After removal of precipitated material all filtrates were made up to 100 per cent saturation and flocculated material recovered. Following dialysis and lyophilization, the penicillinase activity of each fraction was determined. The results are presented in Table II.

From these results it would appear that maximal precipitation of active material is obtained at 70 per cent ammonium sulfate saturation and that at higher salt concentrations increasing amounts of inactive material floc-

culate. A considerable advantage to the selection of the 70 per cent level is that this reduced ammonium sulfate concentration decreases viscosity of the solution and speeds up the filtration process.

Removal of impurities can be effected by saturations of ammonium sulfate from 10 to 40 per cent, while above this level appreciable losses of enzyme occur. The amount of penicillinase removed in the range of 20 to 40 per cent saturation was found to be dependent upon protein concentration, considerable quantities of enzyme being carried down from concentrated solutions. For this reason, a level of 10 per cent ammonium sulfate was adopted as most practicable for general use.

TABLE II

Ammonium Sulfate Fractionation of Acid-Precipitated Penicillinase

The activity is expressed as dilution units per mg. of dry weight.

(NH ₄) ₂ SO ₄ saturation	Activity	(NH ₄) ₂ SO ₄ saturation	Activity*
<i>per cent</i>			
10	256	10% made to 100%	1,000,000
20	256	20% " " 100%	260,000
30	1,024	30% " " 100%	260,000
40	1,024	40% " " 100%	2,048
50	2,048	50% " " 100%	1,024
60	33,000	60% " " 100%	64
70	>1,000,000	70% " " 100%	32
80	1,000,000	80% " " 100%	8
90	1,000,000	90% " " 100%	4
100	1,000,000		

* Apparent enzyme losses near the half saturation level can be accounted for by greater weight of low activity material.

Repeated tests on filtrates after precipitation of enzyme by saturated ammonium sulfate failed to demonstrate residual penicillinase activity, and quantitative removal of enzyme is therefore indicated. This finding is at some variance with the report by Housewright and Henry (2) that less than 10 per cent activity was recovered following precipitation with saturated ammonium sulfate at pH 5.0, 7.5, and 8.8.

Based on the above experimental data the following fractionation procedure was adopted. The acid-precipitated enzyme is dissolved in 0.5 per cent ammonia water to one-fortieth the volume of original culture. Impurities are removed at 10 per cent ammonium sulfate saturation and active enzyme is precipitated at 70 per cent saturation. A period of 1 to 2 hours at 5° was found advisable to permit flocculation with both fractions.

Dialysis—The enzyme was quantitatively dissolved from the filters in distilled water. After transfer to cellophane sacs it was purified by dialysis against distilled water at 5° for a period of 24 hours. It was noted that

dialysis of concentrated solutions resulted in protein flocculation as the salt concentration approached zero.

The dialyzed material was lyophilized and refrigerated over a desiccating agent.

TABLE III
Effect of Dialysis upon Penicillinase Activity

The results are expressed as dilution units multiplied by the dry weight of the material.

Experiment No.	Penicillinase	Total activity*
1	Before dialysis	14,000,000
	After "	>22,000,000
3	Before "	6,250,000
	After "	>66,250,000

* All activities reported are based on 1 liter of cell-free culture filtrate.

TABLE IV
Yields and Activity of Penicillinase Prepared by Acid Precipitation and by Super-Cel Adsorption

The activity is expressed as dilution units per mg. of dry weight.

Purification method	(NH ₄) ₂ SO ₄ saturation	Yield*	Activity per mg.
	<i>per cent</i>	<i>mg.</i>	
Acid precipitation	10	20.0	512
	70	17.0	2,000,000,000
Super-Cel adsorption	50	86.0	128
	100	24.5	250,000,000

* All yields reported are based on 1 liter of cell-free culture filtrate.

To determine whether appreciable losses occurred during the dialysis procedure, aliquots of solutions before dialysis were lyophilized and the activity of this material compared with that of the final enzyme. Results of two typical experiments are presented in Table III.

In all cases total activity after dialysis greatly exceeded the calculated total activity of the undialyzed material. No penicillinase could be demonstrated in the dialyzing water from these experiments. Further, when the dialyzed enzyme was redissolved and assayed in dialyzing water in place of the usual 0.05 M phosphate buffer, no inhibition of penicillinase activity resulted. No explanation for the increased activity after dialysis is offered at the present time. However, further studies are in progress.

This observation of greatly increased activity following dialysis is not in agreement with the recent report by Housewright and Henry (2) that

greater than 50 per cent losses occurred at this step. Earlier work by Woodruff and Foster (4) and by Duthie (1), in which penicillinase from other bacterial species was employed, had also indicated enzyme loss during dialysis.

A comparison of the yield and activity of penicillinase prepared by the acid precipitation method with that of enzyme obtained by the adsorption procedure is presented in Table IV.

DISCUSSION

It is apparent that removal of penicillinase from the culture filtrate by acid precipitation is a much more specific procedure than adsorption on Hyflo Super-Cel, since only relatively small amounts of impurities are later removed by salt fractionation. Moreover, it has been established that the enzyme prepared by this new method is many times more active than the final product prepared by the adsorption procedure.

In all experiments careful checks on enzyme recoveries at each step were maintained, and no serious losses were detected at any point. Total activity of purified enzyme was found to correspond very closely with total activity of the original culture filtrate, and it is therefore felt that practically quantitative recovery of penicillinase has been achieved. This correlation is difficult to establish by the previous method, since amounts of enzyme remaining on the adsorbate can only be determined indirectly from the final yield. Moreover, the acid precipitation method presents considerable advantages owing to its simplicity and greatly shortened recovery time. By this procedure penicillinase of extremely high activity can be prepared within 72 hours.

SUMMARY

Further studies on the production of penicillinase by *Bacillus cereus*, NRRL 569, have been conducted. By the careful regulation of cultural conditions very high enzyme titers have been produced in 48 hours.

Isolation of penicillinase from the culture filtrate has been accomplished by adjustment of the pH to 5.2. Precipitated enzymic material is recovered by filtration through fritted glass Büchner funnels. Impurities are removed at 10 per cent ammonium sulfate saturation. The enzyme is subsequently precipitated at 70 per cent saturation, dialyzed, and lyophilized.

Yields and activity of penicillinase by two methods are compared.

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INHIBITION OF THE ACTION OF TYROSINE DECARBOXYLASE BY PHOSPHORYLATED DESOXYPYRIDOXINE

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A great deal of evidence has been presented to show that the hypertension occurring in certain cases of renal dysfunction may be caused by the inability of the kidneys to remove the pressor amines formed by the decarboxylation of dopa and tyrosine. Bing and Zucker (1) showed that the injection of dihydroxyphenyl-L-alanine into the ischemic kidneys of cats caused a rise in blood pressure. Oster and Sorkin (2) showed that the injection of this substance into humans with essential hypertension produced a similar pressor response. Martin (3) showed that rats fed diets high in tyrosine developed hypertension and that tyrosine was not as toxic in pyridoxine-deficient rats (4).

A consideration of these findings suggested that an inhibition of the decarboxylation of dopa and tyrosine, which would prevent the formation of the pressor amines derived from these amino acids, might be an effective therapeutic measure in cases of renal hypertension.

Tyrosine decarboxylase derived from *Streptococcus faecalis* R was chosen as a convenient vehicle for studying the effects of various inhibitory agents on the decarboxylation of the amino acids in question. It has been shown (5) that the coenzyme of tyrosine decarboxylase is a phosphorylated pyridoxal. Karrer and Viscontini (6) have prepared this substance in crystalline form. Since desoxypyridoxine (7) is known to be an effective inhibitor of pyridoxine, its action on the system was studied in the hope of obtaining an inhibition of decarboxylation.

EXPERIMENTAL

Tyrosine decarboxylase was prepared from *Streptococcus faecalis* R according to the method of Epps (8). The bacteria were incubated for 18 hours at 37° and pH 7 in a medium containing 1 per cent acid casein hydrolysate, 1 per cent glucose, and 1 per cent yeast extract. At the end of this period the cells were centrifuged off, washed with water, and dried with acetone.

The action of the displacing agents on this preparation was studied by the use of the Warburg apparatus. In the main compartment of each cup were placed 1 mg. of the enzyme preparation dissolved in 2 ml. of 0.2

M acetate buffer, pH 5.5, and 0.5 ml. of a solution of the displacing agent used in water. The side bulb contained 0.5 ml. of a suspension of either M/30 tyrosine or M/15 dopa.

After a 15 minute period of equilibration at 28° the manometers were closed and the substrate tipped from the side bulbs into the flasks. The reaction was run for $\frac{1}{2}$ hour.

The results obtained showed that desoxyypyridoxine¹ was entirely ineffective as a displacer in this system in concentrations ranging up to 300 γ per ml.

Since Gunsalus, Bellamy, and Umbreit (9) had shown that pyridoxal was not active as a coenzyme unless it was phosphorylated, it was a natural

TABLE I

Inhibition of Action of Tyrosine Decarboxylase by Phosphorylated Desoxyypyridoxine
Substrate, M/30 tyrosine. Cups 1, 2, and 3 contained 0.5 ml. of desoxyypyridoxine phosphate preparation.

Time	Manometric reading					
	Barometer	Control	Control	Cup 1	Cup 2	Cup 3
	mm.	mm.	mm.	mm.	mm.	mm.
0 min.	150.0	149.0	150.0	150.0	148.8	150.0
30 "	153.8	167.8	168.6	166.0	164.2	165.0
Net weight .. .	3.8	15.0	14.8	12.2	11.6	11.2
QCO ₂ *.		51.4	49.6	39.2	36.7	36.2

* Expressed in c.mm. of CO₂ evolved per hour per mg. of dried enzyme preparation.

assumption that the lack of activity of desoxyypyridoxine as a displacing agent was due to its lack of a phosphate group.

Desoxyypyridoxine was accordingly phosphorylated by the method used by Umbreit, Bellamy, and Gunsalus (5) for the phosphorylation of pyridoxal. 22 mg. of desoxyypyridoxine were dissolved in 2.2 ml. of water and 0.2 ml. of 1:1 NaOH were added. The solution was chilled in an ice bath and 0.1 ml. of POCl₃ plus 0.4 ml. of 1:1 NaOH was added. The mixture solidified, and was warmed until the precipitate dissolved, then chilled again. The addition of the same amounts of POCl₃ and NaOH was repeated four more times, with warming after each addition. After the last treatment, the solution was chilled until small crystals formed throughout the tube, and was then centrifuged while cold. The precipitate was discarded. The supernatant was brought to pH 6 to 7 with HCl and diluted to 25 ml.

¹ Desoxyypyridoxine was obtained through the courtesy of Dr. R. T. Major of Merck and Company, Inc., Rahway, New Jersey.

In the experiments reported 0.5 ml. of this solution was used, containing an amount of the phosphorylated compound corresponding to about 20 γ of desoxypyridoxine.

This preparation was found to be an effective inhibitor of the action of tyrosine decarboxylase. Two different preparations gave average inhibitions of CO₂ evolution of 20 and 35 per cent respectively. A representative set of experimental figures is given in Table I.

Desoxypyridoxine phosphate has been shown to be an effective displacer of pyridoxal phosphate. Since pyridoxal phosphate is believed to be the coenzyme not only of tyrosine decarboxylase but of all the bacterial amino acid decarboxylases, this displacing agent may assume a wide-spread importance as a regulator of bacterial metabolism.

The wide-spread occurrence of pyridoxal phosphate in animal tissues suggests that this compound has some coenzyme function in the animal body, especially in view of the function of the analagous phosphates of thiamine and riboflavin. In this event the possession of an effective displacing agent becomes of manifest importance.

SUMMARY

Desoxypyridoxine is ineffective as an inhibitor of the action of tyrosine decarboxylase.

Phosphorylated desoxypyridoxine displaces pyridoxal phosphate in the tyrosine decarboxylase system.

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A MANOMETRIC METHOD FOR DETERMINING THE KINETICS OF AN ENZYMATIC HYDROLYSIS OF PEPTIDES*

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(Received for publication, March 29, 1947)

Although a number of standard methods are available for determining the rate of hydrolysis of peptide bonds (1-6), no one of these procedures provides the flexibility of operation which characterizes the manometric techniques evolved in the study of the respiratory enzymes. The present procedure was worked out in the hope of providing a manometric method suitable for use in studying the behavior of catheptic enzymes in crude tissue extracts. It is in dealing with such preparations that the greatest difficulties are encountered with titration procedures. The present method depends on the ability of a bacterial decarboxylase to split off carbon dioxide from free L-tyrosine, but not from tyrosine as bound in peptide linkage in the substrate carbobenzoxy-L-glutamyl-L-tyrosine.¹ Thus, a second or "indicator" enzyme is used to record the reaction kinetics of the catheptic enzyme under investigation.

Stimulation for this approach came from recent estimation (7) of the amino acid composition of protein hydrolysates by means of bacterial decarboxylases, and the observation (8) that L-tyrosine decarboxylase will not attack glycyl-L-tyrosine. An analogous method has been used by Zeller (9, 10) in following the distribution of peptidase in the brain. Whereas Zeller's procedure, in which a snake venom amino acid oxidase is employed, is most suitable for dealing with peptidases at pH 7, the method to be described below operates optimally at pH 5.0 to 5.5.

The principle of the decarboxylase-catheptic enzyme method is to be distinguished from that of the urease-arginase method of Weil and Russell (11). In the former the entire course of the enzymatic hydrolysis is followed dynamically, whereas in the latter method, at the termination of the arginase reaction, a single determination of the total amount of urea liberated during that period is made.

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¹ The substrate carbobenzoxy-L-glutamyl-L-tyrosine was obtained through the courtesy of Dr. G. W. Irving, Jr., and the late Dr. Max Bergmann.

Materials and Methods

Preparation of Decarboxylase—The tyrosine decarboxylase was prepared from *Streptococcus lactis* R, obtained from the American Type Culture Collection. The culture medium of Bellamy and Gunsalus (12) was used. The decarboxylase was purified according to the procedure of Epps (8), through stage three, with citrate buffer. At this point, the coenzyme is still attached to the apoenzyme.

For assay purposes, a 20 mg. per cc. concentration of dry powder was suspended in 0.4 M citrate buffer at pH 5.5, incubated 16 hours at 37°, and centrifuged 15 minutes at 2400 R.P.M. The supernatant was either employed immediately or was frozen in a dry-ice box and used a few days later. Addition of synthetic pyridoxal phosphate (13) nearly doubled the activity of the decarboxylase, but the activity was sufficient for present purposes without the addition of this codecarboxylase.

Preparation of Cathepsin—A cathepsin preparation was made from swine kidneys by ammonium sulfate fractionation, according to the procedure of Fruton and Bergmann (14). The active fraction was dissolved in 1 per cent sodium chloride, dialyzed against 1 per cent sodium chloride for 48 hours at 3°, and frozen in a dry-ice box. Enzyme stored under these conditions has maintained a high order of activity for over 3 years.

Manometric Details—L-Tyrosine decarboxylase activity was measured in Warburg manometers by a modification of the procedure of Epps (8). Unless otherwise stated, all reactions were carried out at 25°, in the presence of nitrogen, buffered by 0.20 M citrate buffer at pH 5.4 to 5.5. In some cases the nitrogen was purified by passage over hot copper. Substrate was usually tipped in from the side arm after equilibration. The main compartment contained the cathepsin, decarboxylase, and buffer. The carbon dioxide evolved has been expressed as micromoles per cc. of test solution. All flask readings have been corrected for untipped controls. Since the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by a catheptic “pepsinase” (15) does not require the presence of an activator such as cysteine (16), none was added.

In some cases, the course of the enzymatic hydrolysis was followed in parallel with the manometric method by determination of amino nitrogen, according to the method of Van Slyke (4), or by the titration procedure of Grassmann and Heyde (17). As previously recorded (16), the end-point in the latter titration procedure is difficult with tyrosine-containing substrates.

Results

The relation of the reaction rate to the concentration of cathepsin is shown in Fig. 1. There is a good proportionality between the cathepsin concentration and the measured activity.

The relation of the reaction rate to the concentration of decarboxylase is shown in Fig. 2. In the presence of a large excess of decarboxylase (upper two curves), the reaction rate is relatively independent of the decarboxylase concentration and strictly dependent on the cathepsin concentration. It is therefore desirable to employ decarboxylase concentrations in this range.

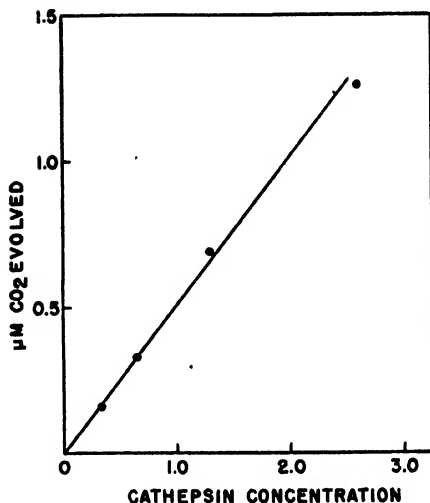


FIG. 1

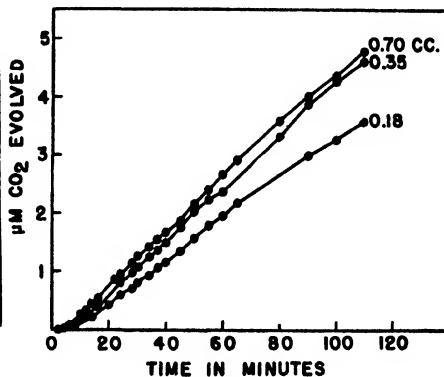


FIG. 2

FIG. 1. Effect of varying the cathepsin concentration on the rate of hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine. Micromoles of CO₂ evolved between 15 and 30 minutes elapsed time after tipping. The cathepsin concentration is expressed in mg. of nitrogen per cc. of test solution. The test solution contained the following: 0.4 cc. of 0.055 M carbobenzoxy-L-glutamyl-L-tyrosine, 0.5 cc. of decarboxylase-citrate, and 0.2 cc. of cathepsin diluted with 0.9 per cent sodium chloride.

FIG. 2. Effect of varying the decarboxylase concentration on the rate of hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by cathepsin. The decarboxylase is expressed as cc. in the test solution. The test solution contained the following: 0.3 cc. of 0.055 M carbobenzoxy-L-glutamyl-L-tyrosine, 0.1 cc. of cathepsin, and 0.7 cc. of decarboxylase-citrate; dilutions made with buffer. The experiment was carried out in 95 per cent nitrogen-5 per cent carbon dioxide.

The rate of reaction of the decarboxylase on L-tyrosine alone is proportional to the decarboxylase concentration, as is shown in Fig. 3.

The relation of the reaction rate to the concentration of carbobenzoxy-L-glutamyl-L-tyrosine is illustrated in Fig. 4. When the initial substrate concentration is above 0.017 M, the reaction rate is relatively independent of the substrate concentration during the first portion of the hydrolysis. $K_s = 3.0 \times 10^{-3}$, as calculated according to Lineweaver and Burk (18). At concentrations above 0.05 M, an inhibiting effect due to substrate ex-

cess begins to appear. The reaction kinetics are discussed in greater detail by Frantz and Stephenson (19).

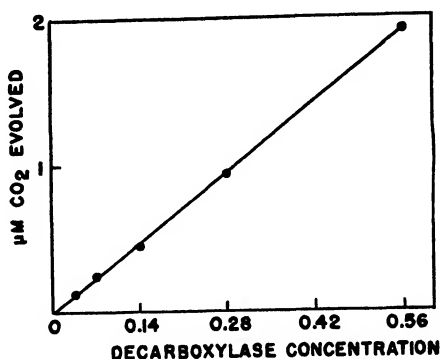


FIG. 3. Effect of varying the decarboxylase concentration on the rate of splitting of L-tyrosine. The decarboxylase concentration is expressed as mg. of nitrogen per cc. of test solution. CO₂ evolved during a 75 minute reaction time. The test solution contained the following: 0.2 cc. of 0.033 M L-tyrosine and 0.6 cc. of decarboxylase-citrate (1.02 mg. of nitrogen per cc.); dilutions made with buffer.

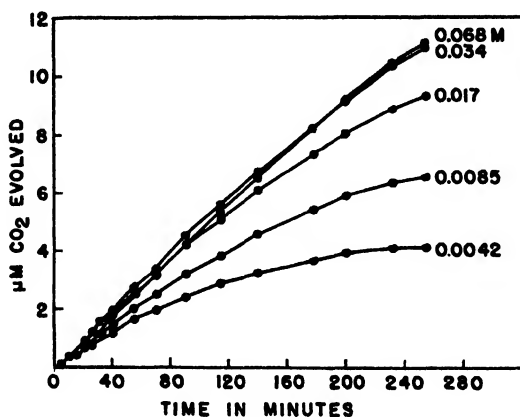


FIG. 4. Effect of varying the concentration of carbobenzoxy-L-glutamyl-L-tyrosine, the concentrations of cathepsin and decarboxylase being kept constant. The figures on the curves indicate the molar concentration of carbobenzoxy-L-glutamyl-L-tyrosine in the test solution. The test solution contained the following: 0.7 cc. of decarboxylase-citrate, 0.1 cc. of cathepsin, 0.3 cc. of carbobenzoxy-L-glutamyl-L-tyrosine at five different concentrations. The experiment was carried out in 95 per cent nitrogen-5 per cent carbon dioxide.

It was previously shown (7) that at pH 5.5 carbon dioxide retention in a tyrosine decarboxylase system was very slight, amounting to less than 5 per

cent. Our results are in agreement with this finding, both on a basis of the theoretical 100 per cent carbon dioxide evolution and as a result of tipping in acid.

Comparison of the manometric method with Van Slyke amino nitrogen (4) determinations, run in parallel on the same type of test solution (but with the omission of decarboxylase), were made. The satisfactory agree-

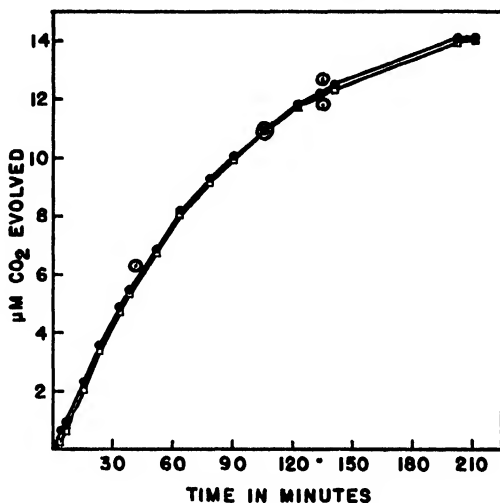


FIG. 5. Comparison of the manometric method with Van Slyke's amino nitrogen method (4) for determining the rate of hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by cathepsin. The amino nitrogen values are converted to micromoles of nitrogen per cc. of test solution. The lines represent duplicate determinations by the manometric method; the four open circles represent amino nitrogen values. The test solution for the manometric determination contained the following: 0.6 cc. of decarboxylase-citrate, 0.3 cc. of 0.055 M carbobenzoxy-L-glutamyl-L-tyrosine, and 0.2 cc. of cathepsin. The test solution for the Van Slyke determination contained 4.2 cc. of citrate buffer, 0.4 M, 2.1 cc. of carbobenzoxy-L-glutamyl-L-tyrosine, and 1.4 cc. of cathepsin (7 times the amounts used in the Warburg vessels, but without decarboxylase).

ment of the two methods is illustrated in Fig. 5. Fig. 5 further indicates that removal of one of the split-products (tyrosine) has no noticeable effect on the rate of the hydrolytic reaction. In order to check particularly on this point, the colorimetric amino nitrogen method of Frame, Russell, and Wilhelmi (20, 21) was used as a further standard. Comparisons were made of the rate of production of tyrosine in this enzymatic reaction as determined manometrically and colorimetrically. Good agreement was obtained between the two methods, and there was no evidence of acceleration of the enzymatic reaction as a consequence of removal of one of the split-products.

The effect of adding the other split-product of peptide hydrolysis, carbobenzoxyglutamic acid, has been studied by Frantz and Stephenson (19). Their results show that carbobenzoxyglutamic acid has an inhibitory effect on the enzymatic splitting of carbobenzoxyglutamyltyrosine. Thus from the hydrolysis of this peptide, one split-product (carbobenzoxyglutamic

TABLE I
Reproducibility of Decarboxylase Method

Manometric details are given in Fig. 5. The figures represent micromoles of carbon dioxide evolved. Theoretical 100 per cent hydrolysis is 15.03 micromoles.

Time elapsed	Flask 1	Flask 2	Flask 3	Flask 4	Flask 5	Flask 6	Standard deviation*
<i>min.</i>							
5	0.32	0.37	0.41	0.35	0.37	0.41	0.047
10	1.14	1.22	1.26	1.18	1.14	1.18	
15	2.07	1.99	2.07	2.04	1.99	2.04	
20	3.01	2.97	3.01	2.88	2.85	2.93	
24	3.70	3.66	3.66	3.62	3.58	3.70	
35	5.57	5.61	5.49	5.53	5.49	5.61	0.100
47	7.35	7.40	7.32	7.28	7.32	7.40	
58	8.45	8.57	8.38	8.42	8.45	8.54	
65	9.15	9.27	9.07	9.20	9.27	9.35	
73	9.84	9.97	9.76	9.83	9.92	9.80	
81	10.43	10.68	10.40	10.65	10.58	10.48	0.083
90	11.25	11.45	11.20	11.35	11.35	11.25	
110	12.30	12.50	12.40	12.48	12.48	12.30	
126	13.00	13.25	13.10	13.18	13.20	13.20	
147	13.80	13.99	13.86	13.95	13.95	13.80	
170	14.28	14.41	14.35	14.35	14.48	14.25	0.102
195	14.65	14.88	14.80	14.70	14.83	14.65	
205	14.70	15.00	14.83	14.90	14.90	14.78	
215	14.90	15.10	14.91	15.00	15.00	14.82	
260	15.18	15.43	15.23	15.31	15.31	15.15	
281	15.18	15.40	15.20	15.30	15.30	15.15	0.102
290	15.20	15.43	15.23	15.31	15.40	15.20	

* $S = \sqrt{\Sigma(x^2)/(N-1)}$ (Arkin and Colton (22)).

acid) inhibits the peptidase reaction, whereas the other split-product (L-tyrosine) does not.

Zeller and Maritz (9) used snake venom amino acid oxidase to measure the rate of hydrolysis of peptides by brain peptidases in the vicinity of pH 7. They reported an acceleration of peptidase activity as a consequence of the removal of amino acid from the reaction mixture by means of the amino acid oxidase. The amino acid oxidase method thus differs strikingly in this respect from the decarboxylase method.

As a test of the reproducibility of the method, six Warburg vessels containing cathepsin, carbobenzoxy-L-glutamyl-L-tyrosine, decarboxylase, and buffer were run in replicate. The results are recorded in Table I.

According to Umbreit *et al.* (23), the over-all accuracy of the Warburg apparatus, as used under conditions similar to the present ones, is within 5 per cent. It may be concluded that the reproducibility of the decarboxylase method is within the limit of accuracy of the apparatus.

As is evident from Table I, the hydrolysis of quantities of substrate of the order of 1 micromole may be followed with satisfactory accuracy.

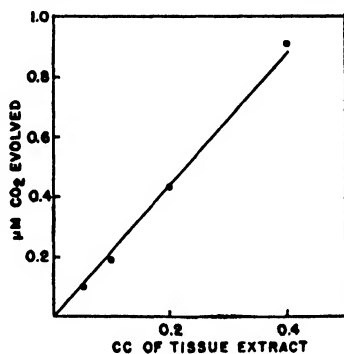


FIG. 6. Effect of varying the concentration of liver extract on the rate of hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine. Micromoles of CO_2 evolved between 6 and 20 minutes elapsed time after tipping. The test solution contained the following: 0.3 cc. of 0.055 M carbobenzoxy-L-glutamyl-L-tyrosine, 0.6 cc. of decarboxylase-citrate, and 0.4 cc. of enzyme diluted with 0.9 per cent sodium chloride. The enzyme solution was prepared by grinding up 450 mg. of lyophilized liver with 5 cc. of 0.9 per cent sodium chloride, centrifuging, and filtering through Pyrex glass wool. In the controls, water instead of substrate was tipped into the reaction mixture from the side arm.

As a test of the method in dealing with a catheptic enzyme as extracted from crude tissue powder, the type of experiment recorded in Fig. 6 was performed. A fairly linear relationship between concentration of tissue extract and enzymatic activity is present during the early phase of the reaction. The limit of sensitivity of the method under present working conditions is in the region of 0.1 micromole (*cf.* Fig. 6). The substrate carbobenzoxyglycyl-L-tyrosine is hydrolyzed by swine kidney carboxypeptidase (15), and the course of this reaction has been followed manometrically. Cysteine, which is an activator for this enzyme, has been found, however, to have an inconstant mild inhibitory effect on the tyrosine decarboxylase system. 0.01 M glutathione also activates this catheptic carboxypeptidase, but does not appreciably inhibit the tyrosine decarboxylase system.

DISCUSSION

The good agreement of results in which decarboxylase is omitted with those in which decarboxylase is present (*cf.* Fig. 5) is evidence that the decarboxylase does not contain substances which activate or inhibit the cathepsin appreciably. Likewise, a concentrate of the supernatant portion of a boiled decarboxylase preparation had no effect on the catheptic reaction.

When the activity of crude cathepsin preparations, as contained in aqueous extracts of tissue powders, is followed by titration procedures, the autolysis blank is found to comprise a disturbingly large fraction of the total titration. The autolysis blank in such a case measures the splitting of numerous types of peptide bonds by a number of enzymes. This difficulty is minimized by the use of the present method, by which the rate of scission of very few types of peptide bonds is measured.

Specific decarboxylases have been found for the natural forms of the amino acids lysine, ornithine, arginine, histidine, and glutamic acid (24). It is therefore theoretically possible to follow the hydrolysis of peptide substrates containing these amino acids by an adaptation of the present method. In agreement with Gale (24), we have been unable to find decarboxylases for glycine or for leucine, using cultures of soil bacteria and of *Proteus vulgaris*.

It should be mentioned that, although some experiments have been carried out over a period of hours, the possibility of an artifact due to bacterial multiplication makes it advisable to rely on short reaction periods or to add a bacteriostatic agent.

While the decarboxylase is used in the present experiments simply as a convenient indicator of the activity of a second proteolytic enzyme, it is worth recalling that both of these enzymes are present in the kidney (25-27). It is thus possible that the present "*in vitro*" coupled reaction sequence may serve as a model for a process which occurs naturally within the cell under certain circumstances, resulting in liberation of the pressor amine, tyramine.

From the finding of Zeller and Maritz (9) on the acceleration of peptidase activity by removal of split-products, and the one above on carbobenzoxy-glutamic acid, the interesting question arises of whether the rate of peptide hydrolysis in the cell may be influenced by the fate of certain amino acid split-products. By remaining in the equilibrium mixture they may slow down the degradative reaction. Their removal by participation in further reactions such as decarboxylation, deamination, or transamination may facilitate continuation of peptide hydrolysis.

SUMMARY

A manometric method has been described for following the kinetics of the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by a preparation con-

taining catheptic enzymes. The method depends on the ability of a bacterial decarboxylase to split off carbon dioxide from L-tyrosine, but not from carbobenzoxy-L-glutamyl-L-tyrosine.

The authors are indebted to Professor Joseph C. Aub for encouragement and interest and to Dr. Ivan D. Frantz, Jr., for performing the Van Slyke amino nitrogen determinations. Dr. Joseph S. Fruton and Dr. Irwin W. Sizer offered helpful criticisms.

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A STUDY OF THE REACTION KINETICS OF A CATHEPTIC ENZYME WITH A DECARBOXYLASE AS INDICATOR*

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Cathepsin activities have frequently been reported in terms of first order reaction constants (1, 2). The assumption has been made that such a procedure is justifiable at the low substrate concentrations usually employed (3). Using a manometric method (4) well adapted to the study of enzyme kinetics, we have found that hydrolysis by swine kidney pepsinase follows a biphasic curve, at substrate concentrations even lower than those usually employed by other workers. The purposes of this paper are to present the evidence for these statements and to analyze further the reactions involved.

EXPERIMENTAL

Preparation of the enzymes and the experimental methods were as previously described (4).

Fig. 1 represents an experiment in which the rates of splitting of two concentrations of carbobenzoxy-L-glutamyl-L-tyrosine were measured by addition of decarboxylase. Fig. 1 illustrates several characteristics of the system. (a) An initial delay occurs at the beginning of the reaction, before the maximum velocity is attained (*cf.* also Fig. 3). This delay appears to be due in part to the time required for the tyrosine concentration to rise to the constant value maintained during the steady state. The delay is minimized by use of a large excess of decarboxylase. (b) In this range of concentrations, the rate is independent of the substrate concentration, and the reaction may be said to follow zero order kinetics. (c) As the reaction proceeds, the rate falls. This fall cannot be due directly to substrate depletion. In Curve A of Fig. 1, the substrate concentration after 220 minutes remains greater than the initial concentration for Curve B, and yet the initial rates are almost identical.

In an attempt to explain the fall in rate, flasks were incubated for varying

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lengths of time, prior to tipping. Fig. 2 shows that no appreciable inactivation of the cathepsin occurred.

Evidence was found, however, that accumulation of one of the split-products causes a decrease in the rate. Fig. 3 illustrates the inhibiting effect of the addition of carbobenzoxy-L-glutamic acid initially. Addition of the other end-product, tyramine, did not alter the rate. Agreement between the decarboxylase and amino nitrogen methods (4) suggests that accumulation of tyrosine has little effect. This observation also provides evidence against the unlikely possibility that the slowing of the reaction is due to a mass law effect, with reversal of the hydrolysis.

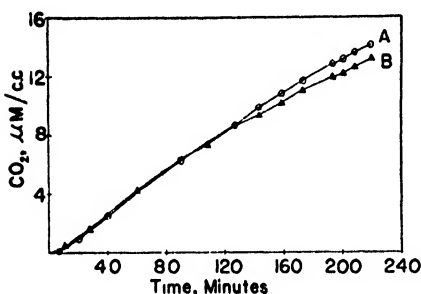


FIG. 1

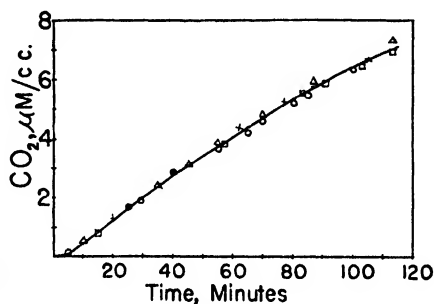


FIG. 2

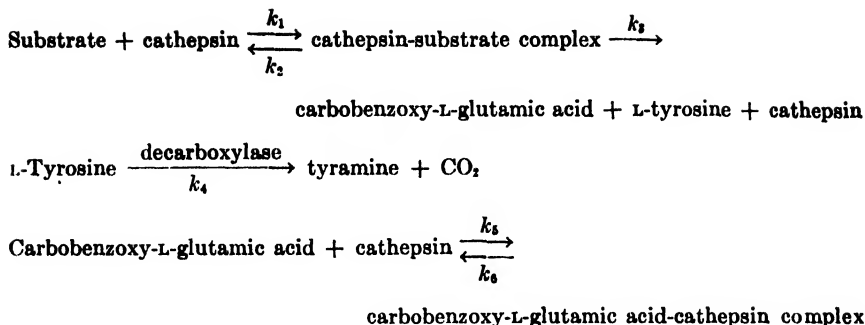
FIG. 1. Course of the reaction at high concentrations of substrate. Swine kidney cathepsin, 0.1 cc. (Curve A), 0.1 cc. (Curve B); decarboxylase, 0.6 cc. (Curve A), 0.6 cc. (Curve B); carbobenzoxy-L-glutamyl-L-tyrosine, final concentration in test solution, micromoles per cc., 58 (Curve A), 29 (Curve B). Total volume in each vessel 1.3 cc.; pH 5.65; temperature 25.0°

FIG. 2. Effect on catheptic activity of incubation at 25°. Composition of solution in each vessel: decarboxylase 0.7 cc; carbobenzoxy-L-glutamyl-L-tyrosine, 0.055 M, 0.3 cc.; swine kidney cathepsin 0.1 cc. The vessels from which the five sets of points were obtained were incubated for varying lengths of time, as follows: O experiment begun immediately, Δ incubated for 30 minutes before the substrate was tipped into the enzyme solution, □ incubated for 60 minutes, X incubated for 150 minutes, ● incubated for 260 minutes.

Fig. 4 shows the course of the reaction for four different concentrations of substrate. In Fig. 5 the equilibrium constants for formation of the enzyme-substrate and enzyme-inhibitor complexes have been evaluated by the graphical method of Lineweaver and Burk (5). The constancy of the ordinate intercept suggests that the inhibition is actually competitive.

Interpretation

The following scheme seems to describe fairly well the reactions which occur in this system.



In the discussion of these reactions, the following symbols will be used.

S = concentration of carbobenzoxy-L-glutamyl-L-tyrosine in micromoles per cc. at time t

S_0 = initial concentration of carbobenzoxy-L-glutamyl-L-tyrosine

t = time in minutes

W = amount of carbon dioxide evolved per cc. of test solution, in micromoles, up to time t

I = concentration of carbobenzoxy-L-glutamic acid in micromoles per cc. at time t

Z = tyrosine concentration in micromoles per cc. at time t

E = cathepsin concentration in arbitrary units at time t

E_0 = initial cathepsin concentration

v = velocity of hydrolysis in micromoles per minute per cc. of test solution

$V = k_3 E_0$, the velocity of hydrolysis when the enzyme is saturated

$$K_s = \frac{k_2 + k_3}{k_1}, \quad K_i = \frac{k_5}{k_6}$$

In the development of this method for practical use, relatively large concentrations of decarboxylase have been used to make the cathepsin activity the dominant factor determining the over-all velocity. The tyrosine concentration is always extremely low. Under these conditions, it seems justifiable to assume that the decarboxylation follows first order kinetics. The differential equation for the final reaction may then be expressed as follows:

$$\frac{dW}{dt} = k_4 Z \quad (1)$$

If we neglect the small amount of substrate tied up in intermediate complexes, the initial substrate concentration should equal the sum of the substrate concentration at time t , the tyrosine concentration, and the amount of carbon dioxide evolved.

$$S_0 = S + Z + W \quad (2)$$

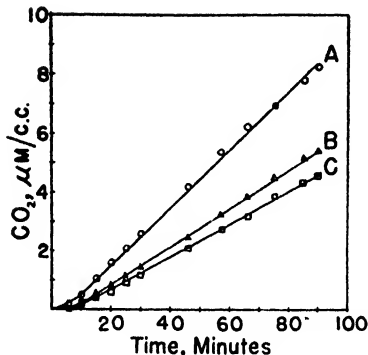


FIG. 3

FIG. 3. Effect of the addition of carbobenzoxy-L-glutamic acid on the activity of cathepsin. The compositions of the solutions in the Warburg vessels were as follows: swine kidney cathepsin 0.1 cc.; carbobenzoxy-L-glutamyl-L-tyrosine, 0.125 M, 0.3 cc.; decarboxylase 0.4 cc.; carbobenzoxy-L-glutamic acid, 0.125 M, 0 cc. (Curve A), 0.15 cc. (Curve B), 0.3 cc. (Curve C). All volumes were made up to 1.1 cc.; pH 5.6; temperature 25.0°.

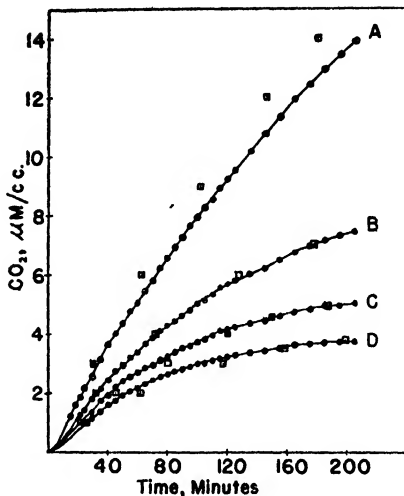


FIG. 4

FIG. 4. Effect of varying substrate concentrations. Substrate concentrations, micromoles per cc., 30.3 (Curve A), 10.8 (Curve B), 6.5 (Curve C), 4.7 (Curve D). Other constituents in each flask: swine kidney cathepsin 0.1 cc.; decarboxylase 0.6 cc. Total volume in each flask 1.3 cc.; pH 5.4; temperature 25.0°. Theoretical points for each concentration are represented by \square , and were calculated by means of equation (12). The values for V , K_s , and K_i were calculated as described under Fig. 5.

Solving for Z and substituting in equation (1), we obtain

$$\frac{dW}{dt} = k_1 S_0 - k_1 S - k_1 W \quad (3)$$

A more convenient form for integration may be obtained by multiplying equation (3) by dt , adding $k_1 W dt$ to both sides, and multiplying by the factor $e^{k_1 t}$ to convert the left hand member to an exact differential.

$$\int (e^{k_1 t} dW + k_1 W e^{k_1 t} dt) = \int k_1 S_0 e^{k_1 t} dt - \int k_1 S e^{k_1 t} dt \quad (4)$$

$$W = S_0 - \frac{\int k_1 S e^{k_1 t} dt}{e^{k_1 t}} \quad (5)$$

Equation (5) furnishes a means of calculating the amount of carbon dioxide evolved, provided we can find S , the substrate concentration, in terms of t and then evaluate the integral. Under certain conditions, these operations are possible. First, let us consider the simplest case, when the

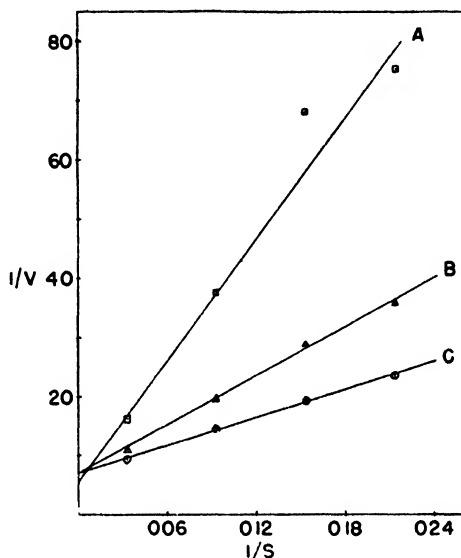


FIG. 5. Evaluation of V , K_s , and K_i . v is expressed in micromoles per cc. per minute and S in micromoles per cc. Concentration of carbobenzoxy-L-glutamyl-L-tyrosine, micromoles per cc., 30.3, 10.8, 6.5, 4.7. Concentration of carbobenzoxy-L-glutamic acid, micromoles per cc., 30.3, 30.3, 30.3, 30.3 (Curve A); 7.58, 7.58, 7.58, 7.58 (Curve B); 0 for each (Curve C). Other conditions were the same as for Fig. 4. $1/V$ is represented by the ordinate intercept. The value 6.5 was used in the calculations, giving 0.154 micromoles per cc. per minute for V ; K_s/V is represented by the slope of Curve C; K_s was calculated to equal 12.6 micromoles per cc., or 0.0126 mole per liter. Sufficient data have not been collected to permit calculation of a statistically significant Michaelis constant. The one arrived at in this experiment happens to be the highest that has been obtained. Experiments with different cathepsin preparations have led to values as low as 0.0041 mole per liter. K_i was calculated from the following relation, $K_s + K_i I/K_i = V$ times the slope of the line. Curve A gives a value of 0.0094 and Curve B 0.0110 mole per liter. For the theoretical points on Fig. 4, the value 0.01 was used.

substrate concentration is so large that the rate of hydrolysis is independent of it and is determined entirely by the cathepsin concentration. Referring to the system of velocity constants originally defined, we may write

$$-\frac{dS}{dt} = k_2 E_0 \quad (6)$$

Integrating and evaluating the constant of integration, we obtain

$$S = S_0 - k_2 E_0 t \quad (7)$$

Substituting this value for S in equation (5), integrating, and evaluating the constant of integration, we find

$$W = k_2 E_0 t - \frac{k_1 E_0}{k_1} (1 - e^{-k_1 t}) \quad (8)$$

Next, let us consider the situation in which the initial substrate concentration is so low that the hydrolysis is first order. Under these conditions, the substrate concentration will fall along a "die away" curve.

$$S = S_0 e^{-k_1 t} \quad (9)$$

Substituting this value in equation (5), integrating, evaluating the constant of integration, and simplifying, we obtain

$$W = S_0 \left[1 - \frac{1}{k_4 - k_1} (k_4 e^{-k_1 t} - k_1 e^{-k_4 t}) \right] \quad (10)$$

Equation (10) is identical with that given by Daniels (6) for consecutive monomolecular reactions.

To describe the conditions actually existing at various concentrations of cathepsin and substrate, we must include both zero and first order terms. Inhibition by one of the split-products must also be considered. As direct an approach as any to this problem begins with an equation given by Lineweaver and Burk (5) for velocity in the presence of a competitive inhibitor.

$$\frac{1}{v} = \frac{1}{V} \left[K_s + \frac{K_i I}{K_i} \right] \frac{1}{S} + \frac{1}{V} \quad (11)$$

A detailed derivation of equation (11) is presented by Wilson (7).

In the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine, the concentration of inhibitor, I , is not constant, but is equal to the amount of substrate decomposed, $S_0 - S$. If we substitute this value for I in equation (11), substitute $-dS/dt$ for v , integrate, and evaluate the constant of integration,

$$t = \frac{K_s K_s + K_s S_0}{V K_i} \ln \frac{S_0}{S} + \frac{K_i - K_s}{V K_i} (S_0 - S) \quad (12)$$

Michaelis and Menten (8), studying the splitting of sucrose by invertase, found that both split-products were inhibitory. They derived an equation which reduces to equation (12) if inhibition by one split-product is neglected.

Equation (12) provides a relation between the time and the amount of substrate hydrolyzed, throughout the biphasic reaction. In the presence

of a large excess of decarboxylase, the amount of carbon dioxide produced may be considered to be equivalent to the amount of substrate decomposed, and the theoretical course of the reaction may be plotted. Fig. 4 shows a comparison of the observed and calculated curves over a limited range of substrate concentrations.

DISCUSSION

It is interesting to notice that equation (12) becomes formally identical with an integrated equation derived by Van Slyke and Cullen (9), if $1/K_i = 0$, implying no inhibition. In the derivation by Van Slyke and Cullen, however, K_i equals k_3/k_1 , k_2 is neglected, and K_i varies with k_3 . In the case of urease, this situation seems to hold. Chance (10), studying peroxidase, was able to measure the three constants directly, and also found k_2 to be very small in comparison with k_3 . Michaelis and Menten (8), on the other hand, assumed that k_3 was negligible in comparison with k_1 and k_2 . Line-weaver, Burk, and Deming (11), studying nitrogenase in *Azotobacter*, deduced evidence in support of the original assumption by Michaelis and Menten. Briggs and Haldane (12) pointed out the fact that K_i actually represents $(k_2 + k_3)/k_1$.

Chance (10) obtained particular solutions of the differential equations of the Michaelis theory by means of the differential analyzer. His theoretical curves differ slightly from those plotted from the equations discussed above in that they include a very brief increased rate at the beginning, while the enzyme-substrate complex is building up to its maximum value. The methods of obtaining general solutions of the differential equations employed above, and by Michaelis and Menten, Van Slyke and Cullen, and Briggs and Haldane, succeed only because the assumption is made that the rate of change in concentration of the enzyme-substrate complex is negligible in comparison with the rate of disappearance of substrate. Obviously this assumption is not valid at the beginning of the reaction.

The behavior of the terms involving K_i in equation (12) is interesting at low values of $S_0 - S$. As $S_0 - S$ approaches 0, each of these terms also approaches 0. By expansion of the logarithmic term in Maclaurin's series, however, it may be shown that the sum of these terms constitutes a second order infinitesimal with respect to $S_0 - S$. This relationship is another way of stating that early in the reaction inhibitory effects due to the split-products are negligible.

SUMMARY

1. The kinetics of the splitting of carbobenzoxy-L-glutamyl-L-tyrosine by swine kidney pepsinase have been studied, with the aid of a tyrosine decarboxylase.

2. The hydrolysis has been shown to follow a biphasic curve, with zero order kinetics early in its course.

3. One of the split-products, carbobenzoxy-L-glutamic acid, has been shown to inhibit the catheptic activity.

4. Over a limited range of substrate concentrations, the experimental curves have been shown to agree fairly well with theoretical curves based on the theory of formation of an enzyme-substrate complex.

The authors are indebted to Professor Joseph C. Aub and to Dr. Paul C. Zamecnik for their interest and encouragement.

The carbobenzoxy-L-glutamic acid used in these experiments was synthesized by Dr. Max Brenner and was kindly furnished by Dr. Paul C. Zamecnik.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF DESOXYRIBONUCLEIC ACID

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Though several useful reagents have been developed to determine the presence and concentration of desoxyribonucleic acid (DNA) (1-3), all have been found to react with yeast nucleic acid and other natural products. Dische in 1944 (4) reported that, in the presence of cysteine and sulfuric acid, DNA gives a stable pink color which is proportional to the concentration of the nucleic acid. The present communication deals with the quantitative application of Dische's reaction to the specific estimation of DNA.

EXPERIMENTAL

Principle—The method depends on the production of a pink color by the reaction of cysteine and sulfuric acid with DNA. Since pentoses do not yield a color reaction with cysteine and sulfuric acid, yeast nucleic acid is unreactive. Therefore, despite the presence of ribonucleic acid (RNA), a direct estimation of desoxyribonucleic acid can be made.

Reagents—

1. 5 per cent cysteine hydrochloride (Eastman Kodak Company) solution in water.

2. 70 per cent sulfuric acid.

3. DNA standard. 0.05 per cent solution of sodium desoxyribose nucleate¹ in water solution kept at 4°. The N:P ratio of the sodium salt was 1:1.66 (theoretical, 1:1.69).

4. RNA standard. 0.05 per cent of sodium ribose nucleate² in water solution kept at 4°. The N:P ratio of the sodium salt was 1:1.69 (theoretical, 1:1.69).

Procedure

To a test-tube are added 0.05 cc. of 5 per cent cysteine hydrochloride, an aliquot of the unknown solution, the volume of which should not be more than 0.5 cc., and 5 cc. of 70 per cent sulfuric acid. The mixture is then

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¹ We are indebted to Dr. Martin Hanig of this Department for a generous sample of sodium thymonucleate.

² Obtained from the Schwarz Laboratories, Inc., New York.

stirred rapidly with a glass rod, and, after standing 10 minutes at room temperature, optical densities are measured at the 490 $m\mu$ wave band with

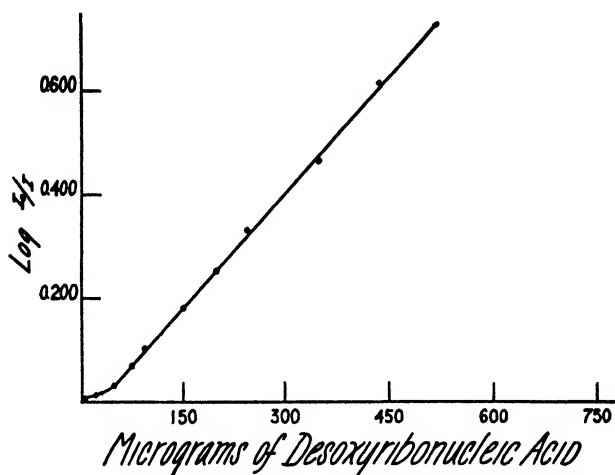


FIG. 1. Relationship between the light absorption at 490 $m\mu$ and the concentration of DNA.

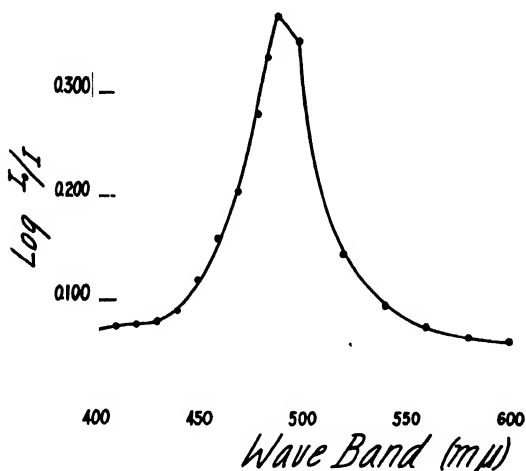


FIG. 2. Absorption curve of the chromogen formed by the interaction of DNA with cysteine and 70 per cent sulfuric acid.

the Beckman quartz spectrophotometer. A reagent blank is used to set the instrument scale to 0.

The concentration of DNA can then be determined by interpolation from

a standard curve prepared by plotting the readings against the known concentrations of DNA as is shown in Fig. 1. However, a standard of about 250 γ of DNA may also be prepared and, from the readings of the standard and the unknowns, the concentration of the latter may be calculated.

As is indicated in Fig. 1, the curve follows Beer's law from a concentration of 25 to 550 γ . Below 25 γ the curve flattens out and above 550 γ the color becomes too intense for accurate readings. Fig. 2 shows that maximum absorption occurs at 490 $m\mu$, and that above and below this sharp peak there is a rapid drop in light absorption. Although the Klett-Sumerson colorimeter can be employed with the 510 $m\mu$ filter, the sensitivity of the method falls considerably.

Factors Involved for Production of Color Intensity

Cysteine—It has been observed that varying the concentration of cysteine changes the color intensity produced by a given concentration of DNA. Optimum concentration of cysteine for maximum color development has been found to be 0.05 cc. of 5 per cent cysteine hydrochloride. As is indicated in Table I, there is a sharp fall if this concentration is not employed.

Concentration of Sulfuric Acid—As is shown in Table II, the color intensity is sensitive to a change in the concentration of sulfuric acid. No color is formed if the acidity is too low, while concentrated sulfuric acid yields a yellow color with DNA. Maximum color intensity is developed when 5 cc. of 70 per cent sulfuric acid are used. The final or critical concentration of acid is, therefore, 63 per cent. Concentrated hydrochloric acid produces no color with the cysteine-DNA system.

Temperature—The determination is carried out at room temperature, which averages between 23–25°. There is little, if any, increase in color if the reaction mixture is incubated for 5 minutes at either 37°, 45°, or 65°.

Stability in Color—The color is unusually stable and does not fade or change. There is, however, a slight increase in color intensity with time. Therefore, it is important to carry out all readings after a standard time interval in order to obtain reproducible results.

Specificity of Method—The method is of considerable value with mixtures of RNA and DNA, since a direct analysis of DNA without interference from RNA can be made. By employing this method together with Bial's reaction (5) for RNA, accurate determinations of both acids can easily be carried out. For example, in an analysis of a known mixture of 150 γ of DNA and 100 γ of RNA, 143 γ of DNA were found directly by this method and 103 γ of RNA by Bial's reaction (after the color intensity contributed by DNA was subtracted from the total green color (at 660 $m\mu$))

Not only is the reagent specific for DNA, but it fails to react with 1 mg. of the following compounds: phosphoglyceric acid, glycerophosphate, glucose-1-phosphate, glucose-6-phosphate, glucose, arabinose, alanine, xanthine, nicotinic acid, coenzyme 1, adenosine triphosphate, and creatine. Fructose and its derivative, fructose-1,6-diphosphate, gave a slight yellow color. However, in any nucleic acid estimation of tissues, the nucleic acid extraction procedure developed by Schneider (6) should be employed to remove interfering substances, such as fructose derivatives.

TABLE I
Effect of Concentration of Cysteine on Color Intensity

Volume of 5 per cent cysteine hydrochloride*	Optical density, $\log \frac{I_0}{I}$	Maximum color intensity
cc.		per cent
0.00	0.000	0
0.01	0.183	59
0.025	0.290	93.5
0.05	0.310	
0.1	0.250	80.7

* 250 γ of sodium DNA, 5 cc. of 70 per cent sulfuric acid, and the indicated amounts of 5 per cent cysteine hydrochloride

TABLE II
Effect of Different Concentrations of Sulfuric Acid on Color Intensity

Concentration of added sulfuric acid*	Optical density, $\log \frac{I_0}{I}$	Color of solution	Maximum color intensity
per cent			per cent
50	0.00		0
60	0.129	Pink	37.5
70	0.345	"	
80	0.332	"	96
90	0.260	Yellow	75
100	0.177	"	52

* 0.05 cc. of 5 per cent cysteine hydrochloride, 250 γ of sodium DNA, and varying concentrations of sulfuric acid to make a total volume of 5.55 cc.

The author is deeply indebted to Dr. Z. Dische for calling his attention to the fundamental observation of the cysteine-sulfuric acid-DNA reaction.

SUMMARY

A specific colorimetric method for the determination of desoxyribonucleic acid has been described. Since ribonucleic acid yields no color with the reagent, desoxyribonucleic acid may be estimated, without interference, in the presence of ribonucleic acid.

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A COMPARATIVE STUDY OF THE METABOLISM OF α -ALANINE, β -ALANINE, SERINE, AND ISOSERINE

II. GLYCOGEN CONTENT OF THE LIVER AFTER ORAL ADMINISTRATION OF THE AMINO ACIDS

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That the administration of protein leads to an increased content of glycogen in the liver, a subject of some controversy in the early part of the present century (1), is now generally accepted. It is also recognized that glyconeogenesis from protein is related to the glyconeogenetic properties of the individual amino acids, components of the protein molecule. The behavior of a considerable number of the natural amino acids as sources of glycogen has been studied. Some of the results have been difficult to interpret as uniform experimental conditions (*e.g.*, age, sex, and weight of the experimental animals, previous diet, period of fasting prior to the administration of the amino acid, period elapsing after administration, etc.) have not been maintained.

Of the amino acids thus studied, the most consistent results have been obtained with alanine¹ in both rats (2-5) and mice (6). The present study is concerned with the content of liver glycogen of young white rats fed D-, L-, and DL-alanine, β -alanine, DL-serine, and DL-isoserine. A study of the results obtained with the three isomers of α -alanine should afford evidence as to the effect of stereoisomeric variation on glycogen formation; comparison of the studies with α - and β -alanine and with serine and isoserine should show any effect of the shifting of the amino group from the α to the β position; and a comparison of α -alanine and serine and of β -alanine and isoserine should reveal the influence of the introduction of a second reactive group (the hydroxyl group) into the molecule of the mono-amino derivatives of propionic acid. This is one of the few studies in

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¹ In earlier work cited (2, 4), the older amino acid nomenclature is used; *d*-alanine of previous papers is the naturally occurring isomer designated in the present paper as L-alanine.

which the glyconeogenetic properties of the various amino derivatives of propionic acid have been investigated under uniform experimental conditions in the same laboratory and by the same workers.

EXPERIMENTAL

The general conduct of the experiments has already been described by us (7) and was essentially the procedure of Cori, as modified in this laboratory (2). The source and purity of the amino acids used have been discussed in our earlier paper (7). Young male white rats of the same stock, whose weight after a 24 hour fast was from 115 to 135 gm., were used. In all cases, determinations of the rate of absorption of the amino acids (7) and the glycogen content of the liver were made with each animal. The period allowed for absorption and glycogen formation was 3 hours. Since alanine is known to be a readily available source of glucose, it was believed that the use of this relatively short period would insure more uniform results. Whether longer periods for absorption and glycogen formation would give results similar to those reported in this paper remains to be determined. In our opinion, the use of more prolonged periods of time (12 hours or more after the administration of the amino acids (5)) introduces complicating factors which we have preferred to avoid for the present.

Immediately after the animals were killed by a blow on the back of the head, the livers were removed, weighed, sliced, and plunged into hot 50 per cent potassium hydroxide solution. Glycogen was determined essentially by the method of Good, Kramer, and Somogyi (8); the glucose obtained by the hydrolysis of the glycogen was estimated by the procedure of Shaffer and Somogyi (9).

The fasting level of the glycogen of the liver was determined for a series of twenty rats of the same stock and receiving the same preliminary treatment as the animals fed the amino acids. The average value of 0.209 per cent was in excellent agreement with the fasting control values previously obtained (2). This series served as a control series for comparison with the results of our experimental series.

The data are presented in condensed form in Table I. The statistical analyses were made through the cooperation of Professor Cecil C. Craig, Director of the Statistical Research Laboratory of the University of Michigan, to whom we wish to express our appreciation.

The oral administration of all the monoamino derivatives of propionic acid studied, with the exception of β -alanine, was followed by a significant increase in the glycogen content of the liver within a period of 3 hours. The magnitude of this increase varied, however. The most marked increases were observed after the administration of DL-alanine. The increase

after administration of the "unnatural" isomer,² D-alanine, was somewhat less, but statistical analyses (Column B, Table I) indicate that the difference between the results with DL- and D-alanine is of doubtful significance. No significant difference between the glycogen of the liver of the control group and of the group receiving β -alanine was observed (Column A, Table I).

TABLE I

Statistical Comparison of Glycogen Content of Liver after Absorption of Amino Acids during 8 Hour Periods

In Column A, the content of hepatic glycogen after the administration of the amino acid under consideration is compared statistically with that of the control (fasted) group; in Column B, with that of the DL-alanine group; in Column C, with that of the D-alanine group; and in Column D, with that of the DL-serine group.

No. of rats	Amino acid	Liver glycogen				† for difference of means			
		Maximum	Minimum	Mean (\bar{X})	S^{*2}	(A)	(B)	(C)	(D)
		per cent	per cent	per cent					
20	Controls	0.359	0.127	0.209	0.00381				
14	DL-Alanine	1.90	0.92	1.42	0.0734	17.1			
18	D-Alanine	1.84	0.41	1.10	0.118	10.5+	2.9 (0.001 <P<0.01)		
17	L-Alanine	0.89	0.28	0.54	0.0241	7.84	10.0	6.10	
13	β -Alanine	0.365	0.056	0.17	0.0126	1.26 (0.2 <P<0.3)			
12	DL-Serine	1.48	0.18	0.83	0.164	5.05	4.5		
12	DL-Isoserine	0.54	0.19	0.41	0.0139	6.21	13.7		3.48 (0.001 <P<0.01)

* Unbiased estimate of the population variance $\left(S^2 = \frac{\sum (X - \bar{X})^2}{N - 1} \right)$.

† The values of t not in bold-faced type are t values as calculated by "Student"-Fisher. For explanation of the values of t in bold-faced type, foot-notes to Table I of our previous paper (7) should be consulted.

The deposition of hepatic glycogen was greater after the feeding of D-alanine than after L-alanine, the natural isomer. The difference was shown

² It is realized that the term "unnatural" isomer may not be entirely justified in view of the presence in certain bacterial (and perhaps other) proteins of considerable amounts of D-amino acids. We have used the term as synonymous with D-amino acid, since the greater part of the amino acids of the molecule of the usual type of proteins is composed undoubtedly of amino acids of the L configuration.

to be statistically significant (Column C, Table I). This result was unexpected and is difficult of explanation. The only comparable series of studies with isomeric alanines with which we are familiar are those of MacKay and his coworkers with white mice (6). D-Alanine was much inferior to L-alanine as a source of glycogen. Moreover, it was noted that racemic alanine and an equimolar mixture of the D and L isomers of alanine were as effective in the augmentation of liver glycogen as was the natural isomer, L-alanine. In our experiments, DL- and D-alanine were almost equally effective, while the glycogen formation after the administration of L-alanine was much less. In our previous experiments (2), the glycogen content of the liver after a 3 hour absorption period was essentially the same with DL- and L-alanine.¹ Both the present results and those of our earlier experiments are contrary to those of Butts and his associates (4), who observed that the liver glycogen after L-alanine was almost double the value observed after DL-alanine. It should be noted that these workers used larger rats, fasted for 48 hours, and that the periods of absorption were 8 rather than 3 hours.

D-Alanine is almost completely converted to glucose in the phlorhizinized dog (10). It is known that tissue slices of the kidney and liver of rats (11-13) deaminate D-alanine more rapidly than L-alanine. Similar evidence in the intact animal is not available. In the frequently cited experiments of Abderhalden and Tetzner (14), DL-alanine was injected *subcutaneously* into rats, and in a single experiment a derivative of D-alanine was isolated from the urine in amounts equivalent to about 16 per cent of the DL-alanine injected or 32 per cent of the D isomer. MacKay, Wick, and Barnum ((6) p. 186) in a foot-note conclude that *in vivo* D-alanine is utilized more slowly in the rat than the L isomer. They also state that after "large" doses of L- and D-alanine were fed to rats, 5 and 27 per cent respectively of the alanine fed were recovered in the urine if all the extra amino nitrogen was assumed to be alanine nitrogen. No determinations of optical activity or isolations were attempted, however.

If D-alanine is deaminized more rapidly than L-alanine, it is possible that the unnatural amino acid is not as readily available for metabolic functions and that pyruvic acid, formed in oxidative deamination, may be rapidly converted to glycogen. More detailed studies of the fate of D-alanine by the use of improved analytical procedures are necessary to clarify this.

The failure of β -alanine to serve as a glycogen precursor was not unexpected in view of the observation that the ingestion of β -alanine by the phlorhizinized dog did not result in extra urinary glucose (15). β -Alanine is not readily deaminized by liver or kidney slices of rats (12).

In confirmation of the observations of Butts and coworkers (16), DL-serine was a good source of liver glycogen, although inferior to DL-alanine (Column B, Table I). The difference between the availability of DL-alanine and DL-serine as precursors of glycogen was not as marked as the difference between their rates of oxidation in experiments with tissue slices (12). With both liver and kidney slices of rats, the rate of oxidative deamination was distinctly lowered by the presence of a hydroxyl group in the β position (12).

It was not anticipated that DL-isoserine would be an important source of glycogen, in view of the observations that it is slowly absorbed from the intestine (7) and that no oxidative deamination by either kidney or liver slices occurred (12). Despite this physiological inactivity in *in vitro* experiments and the poor absorption from the gut, some increase in the liver glycogen was observed, although the values were less than those of any of the other experimental series, except those of animals fed β -alanine. That isoserine may be converted to glucose in the phlorhizinized dog is shown in a single experiment of Kawada, in which extra glucose corresponding to 40 per cent of the theoretical amount (on the assumption that all 3 carbon atoms were converted to glucose) was excreted in the urine (17).

The present experiments include a comparative study of glycogen formation carried out under as nearly uniform experimental conditions as possible. The results are in agreement with earlier observations that the amino derivatives of propionic acid of the protein molecule, alanine (2, 4, 5) and serine (15), are among the most effective of the amino acids in promoting a rapid increase of hepatic glycogen in the fasting rat. The rate of glyconeogenesis has been emphasized by a number of investigators (2, 5, 6). Other factors also play a rôle. The increase of liver glycogen was less when alanine in a gum tragacanth solution was administered than when an aqueous solution was fed (16). Many other seemingly unimportant factors may be concerned. For this reason it is desirable to maintain uniform conditions of experimentation if results are to be compared.

The results obtained in this study with the stereoisomers of alanine are difficult of explanation. They should be repeated and extended if possible. In particular, the use of longer periods of absorption is desirable, provided that proper control conditions are established.

• SUMMARY •

1. The deposition of glycogen in the liver of fasted young white rats after the oral administration of various monoamino derivatives of propionic acid has been studied.
2. The increase of liver glycogen after a 3 hour absorption period may be

listed in the following descending order: DL- and D-alanine (essentially the same), DL-serine, L-alanine, and DL-isoserine. In all these cases, the comparisons with the liver glycogen of control (fasted) animals showed the results to be of statistical significance. The liver glycogen after administration of β -alanine did not differ from the control values.

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A COMPARISON OF THE HOWE AND THE ELECTROPHORETIC METHODS FOR THE DETERMINATION OF PLASMA ALBUMIN

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(Received for publication, May 3, 1947)

During a study in this laboratory on the variations in the total circulating plasma protein and its albumin and globulin components in patients with gastric cancer (Homburger *et al.* (1)), the albumin fraction was measured by both the Howe chemical method (which determines the protein soluble in sodium sulfate (2)) and the electrophoretic method. A review of the literature indicates that the values for the albumin fraction obtained by the Howe method are somewhat higher than those obtained for this component by electrophoresis, but it has been generally assumed that the differences between the results are relatively constant. In this communication is presented an extensive comparison of the albumin concentrations found by these two methods on the same blood samples. The results demonstrate wide fluctuations in the differences between the values and provide data which afford a partial explanation of these fluctuations.

Methods

Blood samples were taken from normal subjects and patients in the postabsorptive state. On one sample total protein and the so called "albumin" by the Howe method were measured. On a second sample the electrophoretic pattern was determined.

For the total protein and chemical "albumin," the blood samples were added to flasks coated with dry sodium citrate. The protein soluble in sodium sulfate was separated by Howe's method, as modified by Robinson, Price, and Hogden (3), to avoid filtration errors. All protein determinations were made by Kjeldahl analysis and were corrected for non-protein nitrogen.

For the electrophoretic analyses, the blood samples were taken into syringes containing 0.1 volume of 5 per cent sodium citrate. The plasma was diluted with buffer to a protein concentration of 2.15 gm. per 100 cc. and dialyzed for 2 days against two lots of buffer. The buffer was 0.06 M in diethylbarbituric acid and 0.008 M in citric acid, with enough sodium hydroxide to bring the pH to 8.6. The ionic strength of this buffer is

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0.098. The mobilities of the plasma proteins are higher in the presence of citrate than in barbiturate alone, and with a potential gradient of 7 volts per cm., an analysis can be made in the long cell in 110 minutes. The percentage of each component agrees closely with that obtained in barbiturate alone, and the boundaries separate more sharply. The boundaries were recorded by the scanning technique of Longsworth (4). Enlarged tracings were analyzed by the method of Svedberg and Pedersen (5). For each component, the values obtained on the ascending and descending sides were averaged.

Because samples of plasma were obtained at irregular intervals, the effect of freezing was investigated. Samples of about 7 cc. were shell-frozen with dry ice and alcohol in small Pyrex bottles, stored in a deep freeze cabinet at -40° , and subsequently thawed rapidly by rotation in water at 30° . No increase in turbidity was observed, and there was no change in the electrophoretic pattern. Samples were, therefore, stored in this fashion routinely. The effect of rapid freezing and thawing on plasma has been discussed by Strumia (6).

In two experiments, a 10 cc. sample of plasma was fractionated by the sodium sulfate procedure. The protein in the filtrate was precipitated by dialysis against saturated ammonium sulfate, redissolved in a small volume of saline, and dialyzed against saline until free of sulfate. It was then dialyzed against the veronal-citrate buffer and analyzed electrophoretically.

Results

In a series of eighty experiments, the sodium sulfate-soluble protein (S. P.¹) values² exceeded the albumin¹ by amounts varying from 1 to 39 per cent. The results are indicated by the hollow symbols in Fig. 1. The solid symbols represent the same S. P. values plotted against the sum of the electrophoretic albumin, α_1 - and α_2 -globulins. These points cluster around the dash line, which is the line of exact equivalence. The S. P. values thus represent the total amount of albumin and α -globulin rather than the albumin alone.

In normal subjects the S. P. values generally include a few per cent of

¹ Although the original definition of plasma albumin was based on solubility, it now seems advisable to reserve the term albumin for that fraction of the plasma protein which migrates as a single electrophoretic component in short time experiments at pH 8.6. The Howe "albumin," which consists of the electrophoretic albumin and variable amounts of globulin, is here referred to as "sodium sulfate-soluble protein," or the "S. P." fraction.

² Since in the normal range small changes in percentage of albumin can cause large changes in the albumin to globulin ratio, the results have been expressed as percentage of total protein.

other protein as well as both α -globulins. That this is probably β -globulin is shown by a study of the proteins included in the S. P. fraction.

Electrophoretic analyses of two plasmas and the S. P. fractions obtained from them are given in Table I. The columns marked "theoretical per cent" show the calculated composition of an S. P. fraction from which the γ -

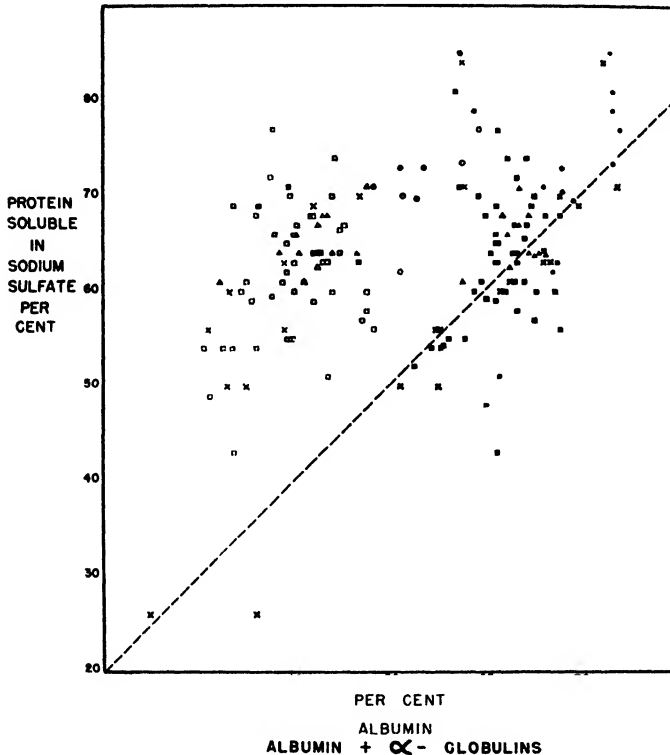


FIG. 1. A comparison of the percentage of the plasma protein soluble in sodium sulfate with the percentage of various electrophoretic components. The hollow symbols are plotted against the albumin alone; the solid symbols are plotted against the sum of the albumin and α -globulins. O, ● = normal subjects; Δ , \blacktriangle = patients with gastric ulcer; \square , \blacksquare = patients with gastric cancer; \times , \times = miscellaneous patients.

and β -globulins and fibrinogen have been removed quantitatively. The observed values are those obtained by electrophoretic analysis of the S. P. fractions of the same plasmas. In Patient 2 (Hodgkin's disease) the expected amounts of each α -globulin and a little β -globulin were found in the S. P. fraction. In the normal subject the S. P. fraction was extremely turbid. The turbidity migrated with the α_1 -globulin; analysis of the

tracings was difficult but the sum of the α -globulins was close to the calculated value. 6 per cent of β -globulin was also present.

In fifteen cases, the plasma proteins were studied on several occasions. These experiments show to what extent the S. P. values reflect changes in the albumin of patients who are being followed during metabolic studies. The results are given in Fig. 2. The first column shows three sets of analyses on subjects on the ordinary hospital diet. The first is a normal subject; the second is a patient with Hodgkin's disease; and the third had lymphosarcoma. In the second column are given the results on three patients with gastric ulcer who received large amounts of protein hydrolysate. In both sets of experiments the S. P. values follow the sum of the albumin and α -globulins closely. Since the amount of α_1 -globulin is small and

TABLE I
Electrophoretic Analysis of Whole Plasma and of Sodium Sulfate-Soluble Fraction

	Normal subject			Patient 2		
	Whole plasma	S. P. fraction		Whole plasma	S. P. fraction	
		Theoretical	Observed		Theoretical	Observed
	per cent	per cent	per cent	per cent	per cent	per cent
Albumin	56.1	78.5	73.1	38.9	58.2	54.9
α_1 -Globulin	6.2	8.7	11.7	11.5	17.2	18.0
		21.6			21.4	
α_2 -Globulin	9.2	12.9	9.7	16.5	24.6	22.7
β -Globulin	12.0		5.6	15.1		4.4
Fibrinogen	5.6		0	4.5		0
γ -Globulin	10.8		0	13.6		0

relatively constant, variations in this component are unimportant. The α_2 -globulin, however, can be markedly increased in the presence of wasting disease, as has been shown by Shedlovsky and Scudder (7), and more recently by Chow (8). Since clinical improvement may be accompanied by a decrease in α_2 -globulin as well as by an increase in albumin, the S. P. value may fail to show any change. This is illustrated best by the case of Patient 2 in Fig. 2; it shows up to a lesser degree in Patients 1, 5, 9, 10, and 12.

A much greater difficulty is illustrated by the results on patients with gastric cancer who received protein hydrolysate. In some cases (Patients 12, 15) the changes in the S. P. value reflect changes in the albumin component quite accurately. In others (Patients 7, 8, 13, 14) the fluctuations in the S. P. values seem to be quite independent of changes in the albumin fraction during the first 20 days of treatment. If this happened only

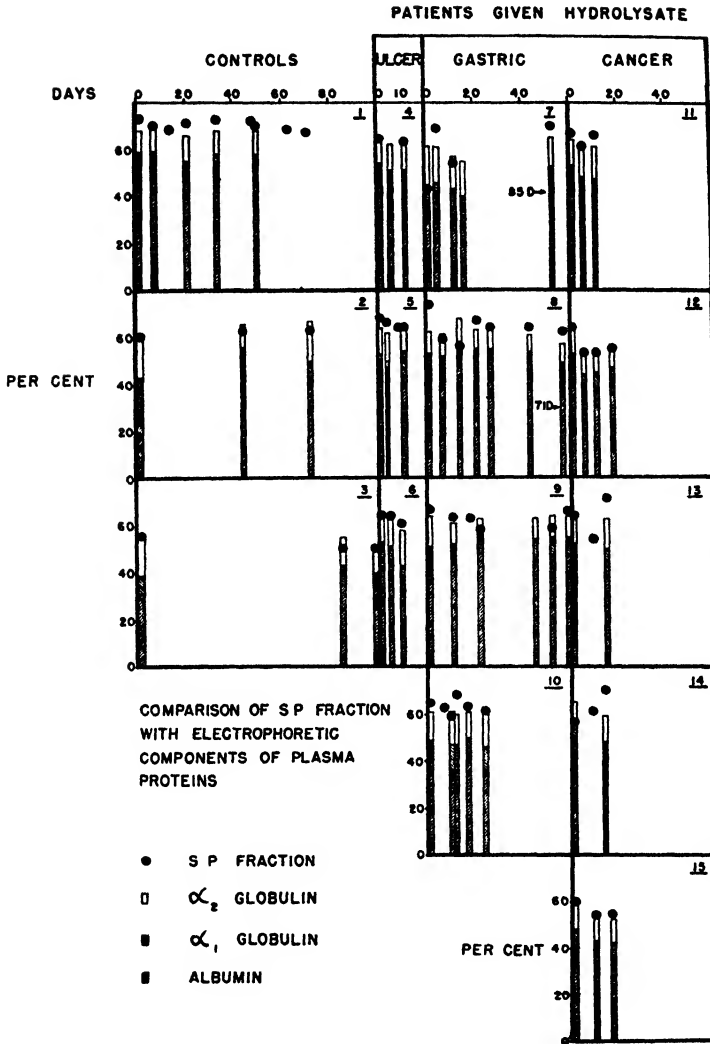


FIG. 2. The changes in the S. P. fraction and in the electrophoretic components of the plasma protein. The ordinate shows percentage of the plasma protein. The abscissa shows time in days. Experiment 7 lasted 85 days, and Experiment 8 lasted 71 days.

occasionally, it might be ascribed to technical error. However, it occurs frequently in patients with gastric cancer and rarely in patients with other disorders. It is apparent that these fluctuations are due to changes in the solubility in sodium sulfate of the plasma proteins of these patients.

DISCUSSION

Although the electrophoretic technique is capable of distinguishing a larger number of plasma protein components than does sodium sulfate fractionation, it does not give absolute values. Three types of errors must be considered. First is the fact that the proportion of albumin obtained on analysis of a given plasma can vary by several per cent, depending on the ratio of buffer to protein concentration in the solution. The theoretical basis for this effect has been discussed by Longworth (9). The buffer salt to protein ratio has been kept constant in these experiments, in order to make the error from this cause as consistent as possible.

A second type of error arises from the assumption that every protein component in the plasma has the same refractive index increment and a nitrogen factor of 6.25. Armstrong, Budka, and Morrison (10) have corrected their electrophoretic analyses on the basis of the refractive index increments and nitrogen contents of the various protein fractions isolated from human plasma. These corrections amount to only 2 per cent, and should be fairly uniform throughout this series of experiments.

The third source of error in the electrophoretic values arises from that portion of the refractive index increments which is caused by the lipides bound to the various globulin components. Some of the purified α - and β -globulins prepared by ethanol fractionation of normal human plasma contain large amounts of cholesterol and other lipides (10). It may be seen from Fig. 2, however, that changes in the total amounts of α -globulin (plus bound lipide) in these patients during the course of hydrolysate therapy are small. Changes in the β -globulin are of the same magnitude.³ If all of these changes were due to lipide alone, the error involved could not account for the inconsistencies between the S. P. values and the albumin found in these experiments.

A summary of the comparative data on determinations of albumin by the Howe and electrophoretic methods found in the literature is given in Table II. When electrophoretic analyses were made at pH 7.4 or 7.8, the values given for "albumin" have been assumed to represent albumin plus α_1 -globulin. In normal human subjects and in many diseases, the S. P. values agree more closely with the albumin plus α -globulin than they do with the albumin alone. Both Gutman and coworkers (12) and Dole (18) have analyzed the S. P. fraction electrophoretically and found α -globulin to be present, and similar results have been obtained in the investigation herein reported.

In patients with cirrhosis (15) or nephrosis (12, 16), the α -globulins are much less soluble. When dogs are depleted of protein by low protein diets

³ Petermann, M. L., and Hogness, K. R., unpublished work.

and plasmapheresis, the α -globulins become less soluble in sodium sulfate (8, 17); when large amounts of liver are fed, the α -globulins become soluble

TABLE II
A Comparison of Howe "Albumin" Values with Electrophoretic Analyses

Authors	Buffer	pH	Subjects	Excess protein in filtrate	
				Over electrophoretic albumin	Over electrophoretic albumin + α -globulins
				<i>per cent</i>	<i>per cent</i>
Taylor and Keys (11)	Phosphate	7.8	8 normal		Average -1.8
Gutman <i>et al.</i> (12)	Phosphate-chloride or veronal	7.4	1 " 1 lymphogranuloma venereum		5
		7.8	myeloma		0
Luetscher (13)	Phosphate	7.8	Normal average		-10 to +19
			Nephrotic		-7.5
			Terminal nephritis		0, -15, -23
			Amyloid disease		+5, -2
			Acute rheumatic fever		-1
			Cirrhosis		-3
Pillemer and Hutchinson (14)	Veronal	8.6	3 normal	Average +7	-1
			7 patients	" +13	
Thorn <i>et al.</i> (15)	"	8.6	Cirrhosis	-2 to +3	-10 to -25
Thorn <i>et al.</i> (16)	"	8.6	Nephrosis	+6 to +19	-4 to -28
Chow (8)	"	8.6	Normal dogs	Average +16	Average -2.5
			Protein-depleted dogs	" +19	" -13
Zeldis <i>et al.</i> (17)	"	8.5	Normal dogs	" +21	" -8
			Protein-depleted dogs	" +27	" -18
Dole (18)	"	8.6	Normals and patients	A:G ratio by electrophoresis = 2/3 A:G ratio by Howe method	

again (17)(see Table II). Recently, Dubach and Hill (19) have shown that, when rabbits are given large amounts of cholesterol, their plasma proteins become less soluble in sodium sulfate. The changes in solubility

follow the rise in blood cholesterol by several weeks, and the authors believe that some slow metabolic change is responsible.

Patients with gastric cancer frequently have impaired liver function (20) and fatty livers (21). The possibility exists, therefore, that in nephrosis or in disorders with impaired liver function, such as cirrhosis and some cases of gastric cancer, the α -globulins contain enough extra cholesterol or other lipide to make them less soluble in sodium sulfate.

When a high intake of protein can be provided for a sufficient time before operation to patients with gastric cancer and impaired fatty livers, the amount of hepatic fat found on liver biopsy is greatly reduced (21). The same effect has been found in patients with gall-bladder disease (22). The lipotropic effect of a high intake of protein or protein hydrolysate may be accompanied by a decrease in the amount of lipide bound to the α -globulins, so that their normal solubility in sodium sulfate is restored. The S. P. value may, therefore, increase, even though the albumin remains constant or decreases.

SUMMARY

1. The fraction of human plasma soluble in sodium sulfate (the "albumin" determined by Howe's method) usually includes the α_1 - and α_2 -globulins as well as the albumin.

2. When a decrease in albumin is accompanied by an increase in the α -globulins, the total amount of protein soluble in sodium sulfate may remain at a constant level. Changes of clinical significance may thus escape detection by this method.

3. In some patients, therapy with protein hydrolysate may cause marked alterations in the sodium sulfate-soluble protein fraction. Since these changes often show no correlation with shifts in the electrophoretic albumin, it is suggested that they are due to alterations in the solubility of the α - and β -globulins in sodium sulfate which are mediated by a lipotropic effect on the liver.

4. It is concluded that the determination of the protein soluble in sodium sulfate by the Howe method may be an unreliable index of the electrophoretic albumin fraction, particularly in patients who are receiving a high intake of protein or protein hydrolysate.

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STUDIES ON ADENOSINETRIPHOSPHATASE IN MUSCLE

I. CONCENTRATION OF THE ENZYME ON MYOSIN*

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Any modern theory of muscular contraction connecting chemical and mechanical events in the functioning muscle will, if it includes the recent work of Engelhardt, Dainty, and Szent-Györgyi, give considerable importance to the identity or close association of the enzyme adenosinetriphosphatase¹ and the muscle protein myosin.

In the announcement of the discovery that the ATPase activity invariably accompanies the myosin fraction of muscle protein, Engelhardt and Lyubimova (2) were careful to state that, although they found ATPase and myosin to be indistinguishable with regard to such properties as solubility, thermolability, and sensitivity to acids, the available evidence was insufficient to determine the identity or non-identity of the enzyme and protein. Subsequent investigators, confirming and amplifying the Russian authors, gave added credence to the implication that the myosin fraction as a whole was identical with the enzyme, although in the negative sense, in that they could effect no separation.

This many sided problem has been ably reviewed recently by Engelhardt (3), stressing the evidence for the identity of myosin and ATPase. There are, however, several aspects of the problem which prompted reinvestigation. Other phosphorylating and dephosphorylating enzymes show in pure form a turnover number, *i.e.* (mole of substrate metabolized per minute)/(mole or equivalent of enzyme), at 38° of 10^4 to 10^6 . If the total myosin represents the ATPase, its maximal turnover number would be about 3×10^2 . This would be even less when actual conditions in the living muscle (pH, ions) are taken into account. The other known and partly purified analogue, the apyrase of potatoes, has a turnover number

* The data in this paper are taken from the thesis to be presented by B. David Polis to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy, when all other requirements have been fulfilled. A preliminary note has appeared (1). We are indebted to the Dazian Foundation for Medical Research and the Penrose Fund of the American Philosophical Society for grants in support of this work.

¹ The following abbreviations are used: ATP, adenosine triphosphate, ADP, adenosine diphosphate, ATPase, adenosinetriphosphatase, apyrase, adenylpyrophosphatase, splitting both labile P groups.

of at least 4×10^5 at 30° (Kalckar (4)). The physical state of myosin, double refraction of flow, viscosity, etc., may be varied considerably without change in enzymatic activity, and may, on the other hand, remain constant while the enzymatic activity may be increased, decreased, or completely abolished (Singher and Meister (5)). Similar lack of correlation is encountered with respect to titratable SH groups (Needham (6)). That the enzyme may be only adsorbed on the myosin is further emphasized by Kalckar's (4) discovery that potato apyrase was easily adsorbed on myosin and that after adsorption it could not be removed. Finally the splitting of ATP by the watery extract of muscle cannot be ignored. The presence of ATPase in the watery extract of muscle was first demonstrated by Lohmann (7). Lyubimova and Engelhardt (8) and also Needham (6) have shown that the splitting occurred with simultaneous phosphorylation of creatine. Whatever this mechanism may be, it can hardly be assumed that the enzyme was dissolved myosin.

The recent work of Szent-Györgyi's laboratory (9), showing that crystallized myosin is still enzymatically active and retains the same activity after repeated crystallizations, cannot be quoted as counter-evidence, because in protein chemistry crystallization does not prove chemical uniformity of the crystals (Meyerhof and Beck (10)).

Our efforts to resolve the situation were begun in the summer of 1944. The procedure developed was briefly described in 1946 (11). On this occasion Price and Cori announced a different method of separation. We were unable to obtain a highly active ATPase free of calcium activation and transphosphorylation by following their procedure. Since then Cori (12) has modified the conclusions drawn from their experiments.

Methods and Units

Following the proposals of Bailey (13), we calculated the activity as Q_P equal to the volume of a hypothetical gas (in microliters) equivalent to the amount of P (in micrograms) liberated by 1 mg. of myosin in 1 hour. The time of incubation was 5 minutes. The myosin protein was determined by the micro-Kjeldahl procedure or, especially for preliminary tests, by the biuret reaction according to Robinson and Hogden (14) or Kingsley (15). The activity was measured with optimal calcium concentration of 1.5×10^{-3} M at optimal pH 9.0 and with at least 4 times as much ATP as was decomposed during the 5 minutes to insure that the reaction ATP-ADP was not inhibited by an accumulation of the end-product.

Myosin was prepared from rabbit muscle. The rabbit was anesthetized with nembutal (10 ml. of saturated nembutal intraperitoneally), bled from the throat, and the cooled muscle excised, ground twice through a cooled meat grinder, and then worked up for myosin according to the procedure

of either Edsall (16) or Szent-Györgyi (9). Double distilled water was used for all extractions.

ATP was prepared from rabbit muscle according to Kerr (17) with some modifications. The alkali treatment and removal of iron by H_2S was omitted, but all traces of the metal as well as barium were removed from the purified barium salt by an ion exchange reaction on Amberlite resin.² For this purpose as much of the barium salt as is necessary for several series of experiments was dissolved in the minimum amount of 0.1 N HCl and washed through a tube filled with Amberlite IR-100 in the proportion of 2 to 3 gm. of the dry resin per 100 mg. of the mono barium salt of ATP used. The resin was washed with double distilled water and the eluate adjusted to the desired pH and diluted to the required volume. Before use the resin must be activated with 4 per cent Na_2CO_3 and then washed free of excess alkali with distilled water. It is then air-dried and stored.

If such a purified ATP is used, the activating effect of glutathione, as described by Ziff (18), vanishes. It has been our experience that glutathione inhibits the enzyme slightly in concentrations higher than 5×10^{-3} M. On the other hand the activation by KCN, as described by Binkley *et al.* (19), still persists.

In a similar manner the advantage of glycine as a buffer also disappears with the use of ATP purified by adsorption on the resin. Thus, while Na_2CO_3 - NaHCO_3 buffer is not suitable, probably because of precipitation of calcium, other buffers, like borate, veronal, and glycine, at optimal concentration of calcium (1.5×10^{-3} M) and optimal pH 9.0 yield the same activity. It appears, then, that the higher activity with glycine found by some authors (Bailey (13)) was due primarily to binding in complex form of toxic traces of metal by the glycine. Clark's (20) 0.2 M borate buffer mixture was used throughout this work.

Final determination of protein was made by the micro-Kjeldahl procedure with a Pregl apparatus having a silver condenser. The distilled ammonia was determined by nesslerization. Comparison with standards was accomplished with an Evelyn photoelectric colorimeter equipped with a filter with maximal transmission at $420 \text{ m}\mu$. The use of alcohol as a protein precipitant, as recommended by Szent-Györgyi, gave such large increases in Q_p of the eluted enzyme compared to the Q_p values obtained when trichloroacetic acid was used as a precipitant that they were considered to be the result of incomplete precipitation.

Absolute Activity of Myosin Preparations—With the same technique, it is a general experience that the activity of myosin preparations is variable within wide limits. Some authors report figures which when calculated

² Amberlite IR-100 is obtainable from The Resinous Products and Chemical Company, Washington Square, Philadelphia.

according to Bailey (13) would give Q_P values as low as 100 to 200 with reprecipitated myosin. Three factors are responsible for these variations. Impurities in ATP, especially heavy metals like mercury and copper, give a very strong inhibition. Second, the age of the myosin preparation when kept at temperatures around 5° has some influence. The activity may decrease appreciably in the first few days, later more slowly. The third factor, not previously mentioned, is the length of time during which the myosin is kept precipitated in distilled water in the procedure of Edsall (16). The longer the myosin precipitate remains in contact with the distilled water, the more it loses its enzymatic activity. After this observation was made, we centrifuged the myosin immediately after dilution and adjustment of the pH to 6.8. The precipitate was washed with water and then redissolved in 0.5 M KCl-0.03 M NaHCO_3 solution. With these precautions a twice reprecipitated myosin generally had an activity of about Q_P 2000, similar to that reported by Engelhardt and Lyubimova (21). Although lower activities occurring between 1000 and 2000 are easily obtained, we have found unfractionated myosin with Q_P values higher than 3000 in only very few instances. One can gather from the papers of Bailey (13), Szent-Györgyi (9), and Engelhardt (3) that values higher than 3000 were also quite exceptional in their work with myosin from rabbits. The Q_P values should be obtained with a turnover of 15 to 60 γ of 7 minute P in 5 minutes and in the presence of about 200 γ of 7 minute P in ATP. With much higher or lower concentrations of total ATP and with the splitting of less ATP, Q_P values may differ somewhat.

A maximal activity of Q_P from 2000 to 3000 in unfractionated myosin is in agreement with the total activity encountered in diluted aliquots of the total muscle mince in KCl at the same pH and calcium concentration, assuming that about half of this protein is myosin. Such an extract gave activities of 1000 to 1200. By addition of 0.01 M KCN the activities were increased about 20 per cent.

Varied fractionation procedures were attempted by several authors. Experiments such as variation of KCl concentrations between 0 and 0.5 M or partial denaturation with urea or guanidine, etc., in all cases showed the myosin activity to change in proportion to the undenatured protein which remained dissolved in KCl solution. We can add to these findings that if the viscosity of myosin was lowered by sonic vibration³ the activity dropped in a similar way.

Q_P Values Obtained with Adsorption on and Elution from Lanthanum Salt—Owing to the intimate connection which apparently exists between the enzyme and the undenatured myosin, drastic treatment of the myosin

³ The oscillator of the Johnson Foundation of Medical Physics, University of Pennsylvania, was used for these experiments.

does not effect a separation. Guided by this principle, we tried different kinds of adsorbents, a method introduced by the school of Willstätter, for partial purification of unstable enzymes. By trial and error it was found that part of the protein was precipitated on the basic lanthanum salt (acetate or nitrate) at pH 8.0. Elution of the enzyme by means of a KCl solution containing KCN and ATP produced fractions which exhibited quite different enzymatic activities, although they all contained myosin. Generally speaking, the fractions rich in enzyme were more soluble than the inert fractions. In those cases, therefore, when the concentration of lanthanum was made to 0.003 M in a 0.2 per cent solution of myosin in 0.5 M KCl and 0.03 M NaHCO_3 , the residual supernatant showed an increased activity. By raising the lanthanum concentration to 0.004 M the protein was almost completely precipitated. Elution of the adsorbed protein with KCl- NaHCO_3 solution containing ATP and KCN yielded fractions showing a 2- to 4-fold increase in activity. The highest absolute activities were obtained when the original myosin itself had an unusually high Q_P , i.e. 4000. In this case values from 10,000 to 15,000 could be obtained.

Procedure

At temperatures not higher than 5°, a highly active fraction of myosin was diluted to a concentration of 0.2 per cent with 0.5 M KCl-0.03 M NaHCO_3 . A 0.1 M lanthanum acetate solution was added dropwise to a solution of myosin, with thorough though gentle stirring, until a final concentration of 0.003 M was reached. Nearly all the protein was in the precipitate that could be centrifuged down. The supernatant solution now had a low concentration of enzyme with activity higher than the original. The precipitate was washed by suspension in KCl- NaHCO_3 solution. After centrifugation, the precipitate was made into a paste with a solution of ATP of such concentration that upon dilution with one-half of the original volume of KCl- NaHCO_3 the final concentration of ATP would be 0.001 M. KCN was added to a final concentration of 0.01 M. The suspension was thoroughly stirred and centrifuged. The supernatant fluid now contained an enzyme with a 2- to 4-fold increase in activity. Repeated elution of the lanthanum precipitate gave enzyme solutions of progressively lowered activity, so that by the third elution the activity of the original myosin was reattained (Experiment 10-3, Table I).

Further purification of the eluted enzyme by repetition of the lanthanum precipitation procedure was unfortunately complicated by the toxic action of lanthanum for ATPase. Not only was there a direct inhibition of the enzyme when lanthanum was added to the incubation mixture, but the eluate on standing, even at low temperatures, rapidly lost its activity and in 24 hours it became negligible. Attempts to remove the lanthanum

by chelation with organic reagents or by combination with heavy metal precipitants such as algin⁴ gave only limited success. Although it was possible many times to obtain an increase in activity 2 to 3 times higher than in the lanthanum eluate alone, the addition of the reagent made the eluate unfit for further purification by a repetition of the lanthanum precipitation (Experiment 1-17, Table I). The inhibiting action of the lanthanum present in the first eluate was counteracted to some extent by the ATP and KCN added. In no experiment was the inhibiting action of lanthanum abolished more than by dilution with borate buffer (Experiment 11-21, Table I).

Elution of the enzyme from the lanthanum precipitate could be accomplished with KCl plus KCN. The enzyme in this instance was usually very dilute, although the activity was increased. The use of ATP released more enzyme from the lanthanum precipitate (Experiments 9-24, 9-27, Table I). This probably was accomplished by interaction of the ATP and lanthanum to form insoluble lanthanum ATP with release of the enzyme. The concentration of ATP in the eluting solution was quite critical. With the use of an excess of ATP it became possible to bring the original myosin back into solution. In general it was quite difficult to obtain enzyme activities with a Q_P over 10,000. Usually the eluted enzyme prepared from Edsall or Szent-Györgyi myosin had activities of Q_P 5000 to 6000.

Since ATP itself has such marked action on myosin, *i.e.* increase in solubility, etc., the question arose as to the necessity for precipitation with lanthanum to effect a type of separation of the enzyme from myosin. Would ATP and KCN alone bring about any kind of increase in activity? In Table I, Experiment 11-21, the activity of the reprecipitated myosin is reported as Q_P 1300. The addition of KCN and ATP to this preparation raised the activity to Q_P 1470. However, after dilution with 0.2 M borate buffer and centrifugation, the activity increased to Q_P 5000. When this myosin with added ATP was subjected to precipitation with lanthanum and subsequent elution, an enzyme with activity Q_P 14,000 was obtained. One might assume that in the presence of ATP the change in salt concentration by dilution with buffer did effect a type of fractionation. If this were so, then one might consider the increase in activity by lanthanum precipitation as 14-fold. Bailey (13), however, reported an activity of Q_P 5000 to 6000 for myosin preparations with no fractionation procedure other than reprecipitation from KCl solution. On the assumption that our extraction procedures were equivalent, at least with respect to ATPase

⁴ Sodium alginate is the purified carbohydrate product extracted from the giant brown seaweeds by the use of dilute alkali. We are indebted to Dr. A. J. Pacini of the Algin Corporation of America for generous samples.

TABLE I
ATPase Activity*

Experi- ment No.	Fraction	Treatment	P split in 5 min	Protein†	Q _p
			γ	γ	
9-27	Edsall myosin	ATP added; centrifuged	33	166 ^b	1,720
	Lanthanum Ppt. 1	Eluted with KCl, KCN†	14	29.6 ^b	4,100
	“ “ 2	“ “ KCl, KCN, ATP	17	29.6 ^b	4,970
9-24	Edsall myosin	ATP added; centrifuged	49	240 ^b	1,700
	Lanthanum Ppt. 1	Eluted with KCl, KCN to 0.02 M	25	23 ^b	9,400
	“ “ 1	Eluted with KCl, KCN to 0.01 M	14	13 ^b	9,500
	“ “ 1	Eluted with KCl, KCN to 0.005 M	14	7.8 ^b	15,200
11-7	Myosin	None	22	101	2,100
	Lanthanum Ppt. 1	Eluted with KCl, KCN	19	62	2,600
	“ “ 2	“ “ “ “	19	43	3,800
11-21	Myosin	ATP Stock diluted 2:5 with buffer	53	350	1,300
	“	KCN added to 0.01 M, ATP to 0.001 M	68	400	1,470
	“	Above diluted 1:4 with borate buffer	53	100	5,000
	Supernatant from lan- thanum ppt.	None	35	146	2,000
	“ “	Diluted 1:5 with borate buffer	18	27	5,350
	Lanthanum ppt.	Eluted with KCl, KCN, ATP; diluted with borate buffer	39	24	14,100
	Myosin	Diluted 1:5 with borate buffer	25	90	2,400
12-3§	Ppt. 1	Eluted with KCl, KCN, ATP	22	42	4,600
	Myosin	KCN added; diluted 1:5 with buffer	16	60	2,300
	Ppt. 1	Eluted with KCl, KCN, ATP	12	29	3,540
	Myosin (12-3)	ATP added; centrifuged	70	600 ^b	1,264
12-5	“ and ATP	Diluted 1:5 with buffer	42	120 ^b	2,270
	Supernatant from lan- thanum ppt.	None	72	284	2,200
	“ “	Diluted 1:5 with buffer	27	47	4,950
	Ppt. 1	Elution diluted 1:5 with buffer	16	28	5,000

TABLE I—*Concluded*

Experiment No.	Fraction	Treatment	P split in 5 min.	Protein†	Q _P
1-17	Crystalline myosin	Diluted 1:10 in borate buffer	16	50	2,780
	Ppt. 1	Eluate diluted 1:5 with buffer	8	29	2,400
	" 2	Eluate diluted 1:5 with buffer and algin	22	20	9,000
10-1	Crystalline myosin	None	57	198	2,600
	Supernatant from lanthanum ppt.	"	14	24	5,050
	Ppt. 1	Eluted from lanthanum ppt. with KCl, KCN, ATP	22	33	5,770
10-3	Twice recrystallized myosin	None	33.8	151	1,950
	Ppt. 1	Eluted with KCl, KCN, ATP	20	29	5,980
	" 1	Second elution with KCl, KCN, ATP	33.8	77	2,780
	" 1	Third elution with KCl, KCN, ATP	22.8	98	2,020

* Activities were measured with 0.5 ml. of borate buffer, 0.5 ml. of 0.02 *N* CaCl₂, 0.2 ml. of 0.01 *M* KCN, and 0.3 ml. of ATP (100 mg. of the mono barium salt per 10 ml.). After mixing, the tubes were brought to 37° and 0.2 ml. of enzyme was added. The reaction was stopped after 5 minutes by the addition of 1 ml. of 10 per cent trichloroacetic acid. The protein precipitate was centrifuged down and PO₄⁻ was determined on 1 ml. of the clear supernatant by the method of Fiske and Subbarow (22).

† All protein determinations were done by the micro-Kjeldahl procedure except those with superscript *b* in which the biuret method was employed.

‡ Unless otherwise stated, KCN is added to a final concentration of 0.01 *M*.

§ No activity in the supernatant of either myosin after lanthanum precipitation.

activity, the increase in activity from 1400 to 5000 was considered as the maximal activation of the unfractionated myosin protein. The 2.8-fold increase after lanthanum precipitation was then taken as an indication of a separation of the enzyme from the myosin.

The use of KCN alone or ATP alone did not increase the activity of the original myosin after dilution with buffer as did the use of both reagents in combination. However, fractionation by lanthanum precipitation still gave a 2.2-fold increase (Experiments 12-3, 12-5, Table I). It is of interest to note that with the addition of ATP to the myosin there was an increase in activity in the supernatant solution after precipitation with

lanthanum. In Experiment 12-3 (Table I) the supernatant alone had no activity, while the supernatant of the same myosin (Experiment 12-5) with ATP gave a doubled increase over the original. This would seemingly indicate that the enzyme combines with ATP, forming a complex that has less affinity for lanthanum than myosin. This enzyme-ATP combination is probably the most promising point of attack for ultimate complete separation of ATPase from myosin.

From the most conservative view-point, there was definitely a 2- or 3-fold increase in enzyme activity over the highest value obtained with the myosin prepared according to the procedure of either Edsall or Szent-Györgyi. As a result we believe these experiments constitute evidence of the non-identity of the protein system called myosin and the enzyme adenosinetriphosphatase.

Inhibition and Activation—Studies conducted on the nature of the inhibiting mechanism for ATPase showed that fluoride inhibited only in that it decreased the concentration of ionized calcium by formation of CaF_2 . Such a conclusion was apparently reached by Engelhardt (3). If manganese was used as the activating metal, no inhibition was observed with F^- concentration up to 10^{-2} N. The maximal activation effect with manganese was, however, appreciably smaller than with calcium.

Barron and Singer (23) found that myosin ATPase was inhibited by chloromercuribenzoate (10^{-3} M). On the other hand Needham (6) and Bailey (13) found no inhibition with iodoacetate. Such findings would be in accord with the generally accepted theory of SH groups of varied accessibility. Iodoacetate attacks only the more superficially located groups which would have nothing to do with enzymatic activity, while the mercuric compounds attack also the less accessible SH groups. Lanthanum may be considered as occupying an intermediate position. While our findings are in general agreement with the results of the above authors, another observation has so far not been mentioned in the literature; low concentration of SH inhibitors, especially the alkylating agents, iodoacetamide, iodoacetate, and iodosobenzoate, and in a lesser degree the oxidizing mercuric compounds,⁵ activate myosin ATPase from about 30 to 80 per cent.⁶ Tables II and III and Fig. 1 give a summary of these experiments.

The experiments of Table II were made without previous incubation of myosin with the SH inhibitors. Since the effect on SH groups is a time reaction, another set of experiments was made in which myosin was in-

⁵ We are indebted to Dr. Hellerman, The Johns Hopkins University, Baltimore, for a gift of most of these compounds.

⁶ Mr. Kielley, Department of Agriculture, University of Minnesota, informs us that he has found in unpublished experiments a similar activation with chloromercuribenzoate on myosin ATPase.

cubated with the SH inhibitors for 10 minutes at room temperature prior to the reaction with ATP. Incubation at 38° was not feasible, since myosin ATPase without ATP was rapidly destroyed at that temperature. In the experiments with previous incubation the tendency to inhibition was much increased. This was due, in part, to the increased concentration of the

TABLE II

Activation and Inhibition of Myosin-ATPase with SH Inhibitors (without Previous Incubation of Enzyme)

Date	Myosin preparation	Substance added	Concentration	P split in 5 min.	Per cent change
			<i>M</i>	<i>γ</i>	
Apr. 30	II	Iodoacetamide	2×10^{-4}	48	
			8×10^{-5}	74	+60
				80	+70
May 2		Iodoacetamide	1.2×10^{-3}	45	
			2×10^{-4}	76	+70
		Iodoacetate	6×10^{-5}	55	+20
			6×10^{-4}	41	-10
" 6				45	0
		Iodoacetic acid		49	
			1.2×10^{-3}	71	+45
			2×10^{-4}	67	+35
" 7	III	Iodoacetic acid	1.2×10^{-4}	67	+35
				43	
			1.2×10^{-4}	63	+50
			2.5×10^{-5}	57	+30
			6×10^{-6}	53	+20
" 8		Iodosobenzoate		43	
			2×10^{-5}	48	+10
			5×10^{-6}	49	+15
		Phenylmercuric hydroxide	1.2×10^{-3}	0	-100
			1.2×10^{-4}	60	+40
" 9		Phenylmercuric hydroxide		54	
			6×10^{-4}	11	-80
			1.2×10^{-5}	69	+30
			5×10^{-6}	68	+30

inhibitor during the time of reaction; *i.e.*, before the inhibitor concentration was lowered by dilution of the enzyme with the testing mixture of ATP and buffer. The increased sensitivity of the enzyme to inhibitors in the absence of ATP also contributed to the enhanced inhibitory effect. Even in this case, the same phenomenon of activation in lowest concentrations and inhibition in higher concentrations was observed.

The activating as well as the inhibiting influence was antagonized by

TABLE III
SH Inhibition Experiments (with Previous Incubation)

Date	Myosin preparation	Substance added	Concentration during previous incubation	Concentration during experiment	Time of previous incubation (room temperature)	P split in 5 min.	Per cent change
			<i>M</i>	<i>M</i>	<i>min.</i>	γ	
Apr. 29	II	Iodoacetate	1×10^{-2}	2.5×10^{-3}	10	47	+20
			2.5×10^{-3}	6×10^{-4}	10	43	-10
		Iodoacetamide	8×10^{-4}	2×10^{-4}	10	85	+80
			3.3×10^{-4}	8×10^{-5}	10	86	+80
May 6	III	Iodoacetamide				31	
			5×10^{-3}	1.2×10^{-3}	10	0	-100
			2.5×10^{-3}	6×10^{-4}	10	3	-90
			8×10^{-4}	2×10^{-4}	10	14	-55
			2.5×10^{-4}	6×10^{-5}	10	22	-30
			1×10^{-4}	2.5×10^{-5}	10	50	+60

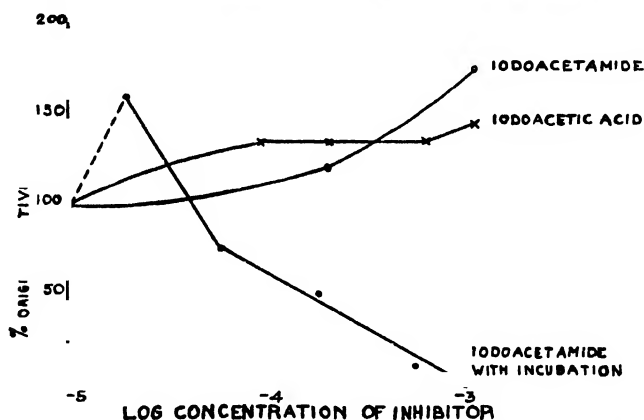


FIG. 1. The effect of SH inhibitors on the splitting of ATP by myosin ATPase

TABLE IV
Effect of Glutathione on SH Inhibition

Date	Added iodoacetamide	Added glutathione	Preliminary incubation	P split in 5 min.	Per cent change
	<i>M</i>	<i>M</i>	<i>min.</i>	γ	
May 10		6×10^{-3}		40	
				25	
	12×10^{-4}	6×10^{-3}		62	+55
	12×10^{-4}	6×10^{-3}		31	+25
	12×10^{-4}		10	23	-42
	12×10^{-4}	6×10^{-3}	10	41	+60

glutathione (Table IV). This was very obvious with iodoacetamide. The chemical interaction of iodoacetamide with glutathione was very slow and incomplete in the absence of the enzyme. It seems, therefore, that the protection of the original activity of the enzyme by glutathione cannot be explained by a chemical reaction between glutathione and the inhibitor substance, but rather by a counteraction of glutathione in reestablishing the SH groups of the enzyme after they are alkylated (or oxidized) by the inhibitor.

It is a pleasure to acknowledge the technical assistance of Mrs. Mildred Miller.

SUMMARY

1. Evidence is presented for the non-identity of the protein system of myosin and the enzyme adenosinetriphosphatase. Primarily this is based on the ability to effect an increase in enzymatic activity of myosin solutions by a mild fractionation procedure. Precipitation of myosin with a basic lanthanum salt and elution with a solution of KCl containing KCN and ATP yield fractions with a 3-fold increase in activity over the original myosin. These activities are at least double the highest reported in the literature for a purified or crystallized myosin.

2. Experiments on the nature of inhibiting mechanisms for ATPase are in general agreement with those reported in the literature, with the addition that low concentrations of SH inhibitors activate myosin ATPase about 30 to 80 per cent. Glutathione was found to counteract both the activating and inhibiting effect.

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INTERMEDIATES IN THE BIOLOGICAL OXIDATION OF ISOTOPIC ACETOACETATE

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In previous reports from this laboratory (1, 2) a study has been made of the intermediates of acetate and acetoacetate oxidation in kidney homogenates. It was found that in kidney homogenates acetate and acetoacetate are oxidized at about the same rate by way of intermediates of the tricarboxylic acid cycle (*i.e.* α -ketoglutarate, succinate, and fumarate). From the distribution of C^{13} in the carboxyl carbons of α -ketoglutarate, formed from carboxyl- and carbonyl-tagged acetoacetate, citrate was excluded as an intermediate of acetoacetate oxidation. This observation has recently been confirmed by Weinhouse, Medes, Floyd, and Noda (3). These latter investigators (4) have also found that acetoacetate is readily formed from acetate in kidney slices and homogenates, even though there is no accumulation of ketone bodies in kidney tissue metabolizing acetate. Medes, Floyd, and Weinhouse (5) have further demonstrated that acetoacetate is probably not an intermediate in the oxidative utilization of acetate.

The question of whether acetate is an intermediate in acetoacetate oxidation, however, remains (6). In the present communication experiments are reported in which a comparison is made of the rate of acetate and acetoacetate oxidation in extracts of rabbit kidney. A study has also been made of the metabolism of isotopic acetoacetate in this extract. Both types of experiments, isotopic and non-isotopic, lead to the conclusion that acetate is not an intermediate of the oxidative metabolism of acetoacetate via the tricarboxylic acid cycle.¹

EXPERIMENTAL

Preparation of Kidney Extract—Extracts of rabbit kidney were prepared by homogenizing 10 gm. of tissue with 10 cc. of cold 0.2 M phosphate buffer (pH 7.6) or 10 cc. of Krebs' phosphate-saline (pH 7.4). This homogenate was then centrifuged at 3000 R.P.M. for 15 minutes at 0° in order to remove the insoluble material. These extracts were cell-free but contained some cellular debris.

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¹ A preliminary report of this work has been published (7).

Methods of Analysis of Organic Substrates—Experiments were usually carried out for 40 minutes at 38° in the presence of oxygen gas. Citrate, acetate, and acetoacetate have been added to these kidney extracts to determine the rates of their metabolism. A study has also been made of the influence of the dicarboxylic acids, α -ketoglutaric and succinic acids, on acetoacetate disappearance, and in some experiments the formation of β -hydroxybutyric acid has also been measured. Citrate was determined by the method of Pucher *et al.* (8) as modified by Hunter and Leloir (9), acetoacetate by the method of Edson (10), and β -hydroxybutyric acid by the method of Weichselbaum and Somogyi (11). The method of Friedemann (12) for the determination of volatile acids has been modified for the determination of small amounts of acetate. When small amounts of acetate (0.05 mm) were distilled according to the procedure of Friedemann, incomplete recoveries of the acid were usually obtained. The quantities of the reagents were consequently reduced 20-fold and the distillation made from a 25 cc. distilling flask. Since under these conditions high blank determinations were often obtained, *p*-toluylsulfonic acid was substituted for sulfuric acid. With this reagent the blank of the determination was reduced and known amounts of acetic acid could be recovered quantitatively. This modification of the method has also been successfully used for the determination of small amounts of acetate in tissue extracts. The procedure used is as follows: 4 cc. of extract were mixed with 4 cc. of water containing 0.056 mm of acetic acid. 0.55 cc. of 10 per cent sodium tungstate was added and followed by 0.7 cc. of 25 per cent *p*-toluylsulfonic acid. The solution should be strongly acid. The precipitate was centrifuged off and washed with two successive portions of 2 cc. of water. The supernatant and washings were transferred to a small distilling flask and the volume of the solution noted. 2.5 gm. of magnesium sulfate and a pinch of mercuric oxide were added and the solution was carefully heated until approximately three-fourths of the volume had been distilled over. Thereupon the precipitate was washed with 2 cc. of water and centrifuged. The supernatant was transferred to the distilling flask and 2 cc. more distilled off. This process was repeated at least three times. The total distillate (about 17 cc.) was then redistilled by the same procedure as above. The second distillate was aerated briefly with CO₂-free air and titrated with 0.01 N NaOH. When 0.056 mm of acetic acid was added to kidney extracts and the distillation carried out as described above, 0.055 mm was recovered in one experiment and 0.057 mm in another.

Isolation of Organic Acids—At the conclusion of the isotopic experiment reported in Table III, acetic and α -ketoglutaric acids were isolated for isotopic analysis. The incubating solution was deproteinized and the residual α -ketoglutarate precipitated as the 2,4-dinitrophenylhydrazone. Acetic acid was then distilled from the supernatant. After redistillation

of the acetic acid, the solution was neutralized and then evaporated to a small volume. It was then acidified with a small amount of nitric acid, silver nitrate was added, and the pH of the solution adjusted to 5.0 with careful addition of ammonium hydroxide. Silver acetate thereupon crystallized out and after recrystallization was washed with alcohol, dried with ether, and placed in a vacuum desiccator over CaCl_2 .

The α -ketoglutaric acid 2,4-dinitrophenylhydrazone was oxidized with acid permanganate to succinic acid which was extracted with ether in the Kutscher-Steudel apparatus. The succinic acid solution was acidified with nitric acid and precipitated as the silver salt after the pH was adjusted to 5.0 with ammonium hydroxide. The silver succinate was then dissolved in a small amount of nitric acid and reprecipitated by the addition of silver nitrate and ammonium hydroxide. Silver acetate and silver succinate were analyzed for their C^{13} concentration in the mass spectrometer.

Results

Metabolism of Various Substrates by Kidney Extracts. Citrate—The addition of sodium citrate to 1 cc. of a phosphate extract of rabbit kidney results in the disappearance of citric acid at a rapid rate (14.0 micromoles in 40 minutes). This extract then differs from the preparation of Hunter and Leloir (9). They have found that washed tissue particles of kidney homogenized in 0.05 M bicarbonate buffer could convert ketone bodies to citrate in the presence of oxalacetate but could not further oxidize the citric acid formed.

Acetoacetate—Although this extract could metabolize acetoacetate with the addition of other accessory substances, the rate could be approximately doubled by adding α -ketoglutarate (Table I). In some instances succinate was effective in stimulating acetoacetate metabolism but the effect of succinate could not be consistently reproduced. The addition of α -ketoglutarate in small amounts does not result in a catalytic increase in acetoacetate oxidation (Table I).

As may be seen in Table II, formation of β -hydroxybutyric acid accounts for approximately 18 per cent of acetoacetate disappearance. As may be seen from the calculations reported in the discussion of this paper, approximately 9 per cent of the disappearing acetoacetate was converted into acetic acid. Thus about three-fourths of the acetoacetate metabolism of kidney extracts takes place by reactions which do not involve simple reduction to β -hydroxybutyric acid or hydrolytic fission to acetic acid. It is presumed that acetoacetate not metabolized in either of these two ways is oxidized by way of the tricarboxylic acid cycle. By means of isotopic acetoacetate it was possible to demonstrate the conversion of acetoacetate to α -ketoglutarate in these extracts.

Comparison of Acetate and Acetoacetate Disappearance—In Table II

is reported a comparison of the rates of metabolism of acetate and acetoacetate in rabbit kidney extracts. It may be seen that ketone bodies are metabolized at about 3 times the rate that acetate is. If these results are expressed in terms of micromoles of acetate and ketone body carbon, approximately 6 times more ketone body carbon than acetate carbon dis-

TABLE I

Effect of α -Ketoglutarate and Succinate on Acetoacetate Metabolism in Extracts of Rabbit Kidney

1 cc. of phosphate extract (pH 7.6) plus 0.6 cc. of additions or a total of 1.6 cc. of solution containing 12.5 micromoles of acetoacetate, 1 mg. of magnesium plus a dicarboxylic acid in the quantity indicated; time of experiment, 60 minutes; oxygen gas.

Experiment No.	Vessel No.	Dicarboxylic acid added	Quantity of dicarboxylic acid added	Acetoacetate disappearance
			micromoles	micromoles
1	1	α -Ketoglutarate	27	11.8
	2	Succinate	34	10.0
2	1	None		3.9
	2	α -Ketoglutarate	2.7	4.9
	3	"	27.0	8.9

TABLE II

Comparison of Rate of Oxidation of Acetate and Acetoacetate by Extracts of Rabbit Kidney

4 cc. of phosphate-saline extract (pH 7.4) plus 4 cc. of additions or a total of 8 cc. of solution containing 220 micromoles of α -ketoglutarate and either 56 micromoles of acetate (Vessel 1) or 53 micromoles of acetoacetate (Vessel 2); time of experiment, 40 minutes; oxygen gas.

Vessel No.	Metabolite measured	Experiment 1	Experiment 2
		micromoles	micromoles
1	Acetate disappearance	10	6
2	Acetoacetate disappearance	30.2	29.2
	β -Hydroxybutyrate formation	5.6	5.6
	Ketone body disappearance	24.6	23.6

appeared. These results then confirm those of earlier investigators who have shown that acetoacetate may be metabolized more rapidly than acetate by certain preparations of kidney tissue (9, 13, 5).

Metabolism of Isotopic Acetoacetate—The problem whether acetate is an intermediate in acetoacetate oxidation has been approached more directly by the use of acetoacetate labeled in the carboxyl and carbonyl positions

with C^{13} . In the experiment reported in Table III isotopic acetoacetate was incubated with kidney extracts along with non-isotopic acetate and α -ketoglutarate. At the conclusion of the experiment the latter two compounds were isolated. Acetic acid contained 0.11 atom per cent excess C^{13} and the succinic acid formed by the oxidation of α -ketoglutaric acid 2,4-dinitrophenylhydrazone contained 0.27 atom per cent excess C^{13} . Thus, the concentration of C^{13} was about 2.5 times greater in the succinate than in the acetate. A better analysis of these data, however, comes from a comparison of the C^{13} concentration of the carboxyl carbons of these compounds. In a previous communication (2) certain conclusions have been drawn concerning the distribution of C^{13} in α -ketoglutarate formed by tissues during the metabolism of isotopic acetoacetate labeled in the

TABLE III

Metabolism of Isotopic Acetoacetate in Extracts of Rabbit Kidney

12.5 cc. of phosphate extract (pH 7.6) plus 12.5 cc. of additions or a total of 25 cc. of solution containing 0.18 mm of acetoacetate; time of experiment, 40 minutes; oxygen gas.

Substance added	Amount added	Amount disappearing	C^{13} concentration (atoms per cent excess)	
			Substance added	Substance isolated
	mm	mm		
Acetoacetate control	0.18	0.103	3.80	
Acetate	0.63		0.00	0.11
α -Ketoglutarate	0.34		0.00	0.27*

* This value represents the C^{13} concentration of succinic acid produced by oxidation with acid permanganate of the isolated α -ketoglutaric acid 2,4-dinitrophenylhydrazone.

carboxyl and carbonyl positions. It is known that the 4 carbon atoms of α -ketoglutarate which yield succinic acid upon acid permanganate oxidation contain most of the C^{13} of the α -ketoglutaric acid molecule. It is further believed that all of the isotope of this succinic acid fraction is present in 1 carboxyl carbon only and that this carboxyl carbon is derived from the γ -carboxyl carbon of α -ketoglutaric acid. The C^{13} concentration of the γ -carboxyl carbon of the α -ketoglutaric acid is thus probably 4 times that of the 4 carbon atoms of succinic acid or 1.08 atoms per cent excess C^{13} . Likewise, acetate produced from isotopic acetoacetate labeled in the carboxyl and carbonyl positions should contain C^{13} in the carboxyl carbon only. The C^{13} concentration of this carbon atom of acetate isolated at the conclusion of the experiment in Table III is then 2×0.11 or 0.22 atom per cent C^{13} . It is thus seen that the C^{13} concentration in the γ -carboxyl

carbon of α -ketoglutaric acid is 5 times greater than that of the carboxyl carbon of acetate.

DISCUSSION

Although it is recognized that acetate is closely related to an important intermediate of both fat and carbohydrate metabolism, it is generally believed that some active form of acetate is the actual intermediate of this metabolism. Before we continued further investigations on intermediates of acetoacetate metabolism, however, it seemed advisable to ascertain the rôle which acetate might play in these reactions. Since the presence of cell membranes may interfere with the interpretations of experimental data, a crude cell-free extract of kidney was prepared which was capable of metabolizing acetoacetate at a relatively rapid rate. This extract also metabolized acetate but at a much slower rate than acetoacetate. This difference in the rate of metabolism of acetate and acetoacetate is contributory but not conclusive evidence that acetate is not an intermediate in the oxidation of the latter. It is known, for example, that the concentration of substrates may play an important part in the rate of their metabolism and it is possible that optimal conditions for acetate metabolism have not been used in these experiments. Furthermore, there is no conclusive evidence that acetate and acetoacetate metabolism in this extract may be accounted for solely by oxidative reactions of the tricarboxylic acid cycle.

The experiment with isotopic acetoacetate avoids these possible criticisms, however. When isotopic acetoacetate is metabolized by cell-free extracts in the presence of large amounts of non-isotopic acetate and α -ketoglutarate, the acetate isolated at the conclusion of the experiment should contain a higher C^{13} concentration than α -ketoglutarate if it is an intermediate of acetoacetate oxidation via the tricarboxylic acid cycle. Such, however, was not the case. In all probability the γ -carboxyl of α -ketoglutarate contained a C^{13} concentration 5 times greater than that of the carboxyl carbon of acetate. Although acetate is thus not a direct intermediate in acetoacetate oxidation, some may be formed during the metabolism of this latter compound. Since, as is shown in separate experiments (Table II), the concentration of added acetate remains relatively constant during the experiments with kidney extracts, it is possible to calculate the amount of acetoacetate converted to acetate. In the isotopic experiment reported in Table III, 0.103 mm of isotopic acetoacetate containing 3.80 atoms per cent excess C^{13} was metabolized. 0.63 mm of acetate was present at the beginning of the experiment and, in view of the experiments reported in Table II, it is unlikely that this concentration changed materially. The concentration of C^{13} in the acetate at the con-

clusion of the experiment was 0.11 atom per cent C^{13} . The quantity of acetoacetate converted to acetate is thus $(0.63 \times 0.11)/(2 \times 3.80) = 0.0091$ mm of acetoacetate. This is equal to 8.8 per cent of the total acetoacetate which disappeared during the experiment. Lehninger (6), who has previously reported the enzymatic splitting of acetoacetate to acetate by kidney extracts, found that acetate formation accounted for approximately 25 per cent of the acetoacetate disappearance in this preparation. His experiments were done, however, in the absence of α -ketoglutarate, a substance which might possibly divert the metabolism of acetoacetate to oxidative channels and thus reduce the amount of acetate formed by purely hydrolytic processes.

Thus, if the fission of acetoacetate into a 2-carbon compound takes place during the initial steps of its oxidative reactions, this 2-carbon intermediate may possibly resemble acetate in chemical constitution but probably is not acetate itself. The small amount of isotopic acetate formed from acetoacetate may result from a side reaction between acetate and some active 2-carbon intermediate of acetoacetate oxidation.

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SUMMARY

Extracts of rabbit kidney have been prepared which are capable of oxidizing acetoacetate and, to a much less extent, acetate. The rate of acetoacetate oxidation may be considerably increased by the addition of α -ketoglutarate but no catalytic effect of α -ketoglutarate on acetoacetate oxidation could be observed. Citrate is also readily metabolized by this extract.

When isotopic acetoacetate is incubated aerobically with non-isotopic acetate and α -ketoglutarate in rabbit kidney extract, the acetate isolated at the conclusion of the experiment contained much less C^{13} than did the α -ketoglutarate. These data indicate that acetate is not a direct intermediate in the oxidative utilization of acetoacetate via the tricarboxylic acid cycle.

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A STUDY OF THE MECHANISM OF FATTY ACID OXIDATION WITH ISOTOPIC ACETOACETATE

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The recent study by Weinhouse, Medes, and Floyd (1) of the oxidation of carboxyl-labeled fatty acids to acetoacetate by rat liver slices has rendered support to MacKay's theory of β oxidation-condensation. These investigators have found that acetoacetate formed from the oxidation of carboxyl-labeled octanoic acid contained isotopic carbon distributed equally in the carboxyl and carbonyl carbon atoms. The hypothesis of MacKay and his coworkers (2, 3) that fatty acids are oxidized by successive β oxidation to 2-carbon fragments which condense randomly to form acetoacetate would predict such an equal distribution of C^{13} between the carboxyl and carbonyl carbons of acetoacetate formed from carboxyl octanoate. Acetoacetate produced from carboxyl-tagged octanoate by a process of multiple alternate oxidation should theoretically contain isotope only in the carboxyl position.

Although the experiments of Weinhouse *et al.* (1) may best be interpreted in terms of the theory of successive β oxidation plus condensation of 2-carbon fragments, it has also been recognized that there are alternate mechanisms of fatty acid oxidation which might possibly explain the appearance of C^{13} in the carbonyl position of acetoacetate formed during the oxidation of carboxyl isotopic octanoate (4, 5). One such alternate hypothesis is that carboxyl-labeled octanoate is oxidized in liver slices by the process of multiple alternate oxidation with the formation of equal amounts of non-isotopic acetoacetate and acetoacetate labeled in the carboxyl carbon only. If this latter species should break down rapidly into 2-carbon fragments which recondense, acetoacetate might be formed, containing C^{13} equally in the carboxyl and carbonyl positions. Although both hypotheses explain the appearance of C^{13} in both the carboxyl and carbonyl position, the reaction by which this distribution takes place would be fundamentally different. In the first theory the formation of a 2-carbon fragment is an integral part of the oxidation process; in the second theory the formation of a 2-carbon compound would result from a side reaction of acetoacetate. In order to determine whether acetoacetate

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can break down into 2-carbon fragments which recondense, two types of isotopic acetoacetate have been synthesized, namely $\text{CH}_3\text{C}^{13}\text{OCH}_2\text{COOH}$ and $\text{CH}_3\text{COCH}_2\text{C}^{13}\text{OOH}$, and incubated with rat liver slices. The results of these experiments follow.

EXPERIMENTAL

Organic Syntheses—The two types of isotopically marked acetoacetate were prepared according to procedures described by Sakami, Evans, and Gurin (6). These procedures consisted of standard organic reactions modified for the synthesis of isotopic compounds in small quantities. Acetoacetate labeled in the carbonyl position was made from carboxyl isotopic methyl acetate and non-isotopic ethyl bromoacetate (7). Carboxyl-labeled acetoacetate was likewise synthesized from non-isotopic methyl acetate and carboxyl isotopic ethyl bromoacetate. This latter compound was prepared by a modification of the procedure of Auers and Bernhardt (8).

Incubation of Tissues—Experiments with rat liver slices were carried out under conditions similar to those described by Weinhouse, Medes, and Floyd (1). Approximately 4 gm. of rat liver slices were incubated with an isotopic substrate in 40 cc. of solution aerobically for 2 hours. At the conclusion of the experiment, samples were removed for the determination of acetoacetate (9) and, in some experiments, β -hydroxybutyric acid (10). The remainder was then divided into two parts. One part was acidified with sulfuric acid and the acetone formed from the acetoacetate upon heating was steam-distilled into a solution of Denigès' reagent and precipitated as the mercury complex. The other part of the incubation solution was acidified with citric acid and any CO_2 thus liberated was removed by aeration of the solution with CO_2 -free air. Thereupon aniline citrate was added and CO_2 produced by the breakdown of acetoacetate was collected in barium hydroxide. Isotopic analysis of this barium carbonate for C^{13} is then a measure of the C^{13} concentration of the carboxyl carbon of the acetoacetate. The C^{13} concentration of the isolated mercury-acetone complex when multiplied by 3 is equal to the C^{13} concentration of the carbonyl carbon of acetoacetate. Weinhouse *et al.* (1) have demonstrated that all of the C^{13} found in acetoacetate formed from carboxyl isotopic octanoate is present in the carboxyl and carbonyl carbons and none in the remaining 2 carbon atoms. All results are expressed in terms of atoms per cent excess C^{13} .

Results

Experiments with Isotopic Acetoacetate—Three experiments, one with carbonyl-labeled acetoacetate and two with carboxyl-labeled acetoacetate,

are included in Table I. In each experiment the initial and final amounts of acetoacetate present in the reaction vessel were measured and are recorded. In Experiment 3, Table I, β -hydroxybutyric acid was also measured at the conclusion of the experiment and it was thus possible to calculate the quantity of total ketones formed.

Measurement was made of the C^{13} concentration of the carboxyl and carbonyl carbons of acetoacetate at the beginning and end of each experiment. Within the limit of experimental error no evidence could be found to indicate that acetoacetate had split into 2-carbon fragments which could randomly recondense, forming acetoacetate again. In Experiment 1 with carbonyl-labeled acetoacetate the concentration of C^{13} in the carboxyl carbon (0.00 atom per cent excess) did not significantly change during the

TABLE I

Experiments with Carbonyl- and Carboxyl-Labeled Acetoacetate on Rat Liver Slices

	Carbonyl-labeled acetoacetate		Carboxyl-labeled acetoacetate			
	Experiment 1		Experiment 2		Experiment 3	
	mm	C^{13} per cent excess	mm	C^{13} per cent excess	mm	C^{13} per cent excess
Acetoacetate (initial)	0.130		0.130		0.084	
Carboxyl carbon		0.00		2.07		2.07
Carbonyl "		1.20		0.39		0.39
Acetoacetate (final)	0.148				0.129	
Carboxyl carbon		0.02		1.33		Lost
Carbonyl "		0.78		0.24		0.30
Total ketones					0.150	

experiment. The concentration of C^{13} in the carbonyl position decreased from 1.20 to 0.78 atoms per cent excess, but this probably is due to dilution of isotopic acetoacetate by non-isotopic acetoacetate formed from endogenous sources of the liver slice. The carboxyl-labeled acetoacetate used in Experiments 2 and 3 (Table I) contained a relatively small amount of isotope also in the carbonyl carbon. This appearance of isotope in the carbonyl carbon results from the self-condensation of isotopic ethyl bromoacetate when conditions for the synthesis of carboxyl-labeled acetoacetate are not strictly observed. The concentration of C^{13} in the carbonyl carbon was only 19 per cent of that in the carboxyl carbon and hence did not interfere materially with the metabolic experiments. If in liver slices there had been redistribution of isotope between the carboxyl and carbonyl carbons, the ratio of isotope in these 2 carbon atoms should have decreased from the original ratio of 5.3 and approached the ratio of 1 as complete redistribution was reached. In Experiment 2 the ratio of C^{13} in the

carboxyl and carbonyl carbons was 1.33/0.24 or 5.5. Both atoms had been diluted by non-isotopic acetoacetate formed from non-isotopic metabolites, but this dilution was the same for either carbon atom. In Experiment 3 a comparison was made of the total ketone bodies formed and the degree of dilution of C^{13} of acetoacetate during the experiment. Unfortunately the sample of CO_2 representing the carboxyl carbon of the final acetoacetate was lost. There is, however, roughly a proportionality between the dilution of C^{13} in the carbonyl carbon and the production of ketone bodies by the liver slice. An accurate comparison of this kind cannot be made from values of the carbonyl carbon, since the absolute error in the determination of C^{13} in low concentrations (*i.e.* 0.10 atom per cent excess) is relatively great. Experiments with both types of aceto-

TABLE II
Experiments with Carboxyl-Labeled Octanoate on Rat Liver Slices

	Experiment 1		Experiment 2	
	<i>mM</i>	<i>C¹³ per cent excess</i>	<i>mM</i>	<i>C¹³ per cent excess</i>
Octanoate (initial)	0.400		0.400	
Carboxyl carbon		7.68		7.68
Acetoacetate (initial)	0.107		0.100	
Carboxyl carbon		0.00		0.00
Carbonyl "		0.00		0.00
Acetoacetate (final)	0.110		0.175	
Carboxyl carbon		0.91		0.98
Carbonyl "		0.63		0.60
Total ketones	0.150			

acetate are in agreement. In neither case could evidence be found that in liver slices there is a measurable splitting of acetoacetate into 2-carbon fragments which randomly recondense.¹

Experiments with Carboxyl Isotopic Octanoate—In Table II are recorded the data of two experiments in which carboxyl isotopic octanoate and non-isotopic acetoacetate were incubated together with liver slices aerobically for 2 hours. Non-isotopic acetoacetate was added at the beginning of the incubation in order to simulate as nearly as possible the conditions of the experiments with isotopic acetoacetate reported above. It was felt that in all experiments the average concentration of acetoacetate during the 2 hour incubation period should be approximately the same in

¹ Although no evidence could be found for the redistribution of isotope between the carboxyl and carbonyl carbons of acetoacetate when liver slices were incubated with either of the two types of isotopic acetoacetate, preliminary experiments have indicated that this reaction may take place in rat kidney slices to a significant extent.

order to compare the results of the experiments with isotopic octanoate and isotopic acetoacetate. Aside from the inclusion of non-isotopic acetoacetate in the incubating medium, the conditions of our experiments with isotopic octanoate were identical with those of Weinhouse *et al.* (1). In contrast to the experiments of these latter investigators we did not obtain equal amounts of C^{18} in the carbonyl and carboxyl carbon atoms of acetoacetate derived from carboxyl isotopic octanoate. In our experiments the carboxyl carbon contained on an average 0.94 atom per cent excess C^{18} , while the carbonyl carbon contained 0.62. Although differing perhaps in quantitative aspects, our experiments with octanoate confirm those of Weinhouse *et al.* which demonstrated that the carboxyl carbon of octanoate is an important precursor of both the carboxyl and carbonyl carbons of acetoacetate in rat liver slices.

DISCUSSION

The experiments reported with isotopic octanoate and acetoacetate in rat liver slices have demonstrated that the isotope appearing in the carbonyl position of acetoacetate formed from carboxyl isotopic octanoate does not arise by a preliminary formation of carboxyl isotopic acetoacetate and a secondary splitting of this compound into 2-carbon fragments which recondense randomly to form the carboxyl- and carbonyl-labeled acetoacetate. On the contrary, these data might favor the point of view that 2-carbon fragments are split off from fatty acids and, failing to be oxidized, are condensed into acetoacetate. This conclusion has been indicated not only by the experiments of MacKay *et al.* (2, 3) and Weinhouse *et al.* (1) but also by those of Lehninger (11). Lehninger has shown that washed liver particles treated with malonate may oxidize octanoate or pyruvate, with the quantitative formation of acetoacetate. Upon addition of fumarate to this system the formation of acetoacetate was reduced and there was an increase in citrate formation when compared to suitable controls. It was furthermore found that acetoacetate could not be oxidized by this enzyme system either in the presence or absence of fumarate. Lehninger has interpreted these data as indicating that acetoacetate is not the condensing agent in citrate formation but rather some 2-carbon compound derived from the oxidation of octanoate or pyruvate.

The experiments of the above investigators thus emphasize the metabolic inertness of acetoacetate in liver tissue and indirectly support the point of view that 2-carbon fragments are the metabolically active products of fatty acid catabolism. Although this point of view may be at present favored over others, it must be accepted with some reservation until more direct evidence is available describing the exact chemical composition and metabolic rôle of this 2-carbon intermediate and until other possible

reaction products of fat oxidation are investigated. It is possible, for example, that not acetoacetate but some active form of acetoacetate is the immediate product of fatty acid catabolism. Thus the experiments of Lehninger could be explained by assuming that an activated acetoacetate rather than a 2-carbon compound plays the central rôle in the oxidation of pyruvate and octanoate and in the formation of citrate from the metabolic products of these substances in liver tissue. Likewise, the appearance of C^{13} in the carbonyl carbon of acetoacetate formed from carboxyl isotopic octanoate may result from preliminary formation of activated acetoacetate isotopic in the carboxyl position and from a secondary splitting of this latter compound into 2-carbon fragments which recondense randomly.

As has been pointed out recently by Stadie (4), the experiments of Weinhouse *et al.* (1), although clearly demonstrating the formation of 2-carbon intermediates in liver tissue, do not necessarily define the mechanism of the reactions whereby this 2-carbon compound is formed from octanoic acid. Stadie (4) has proposed, for example, that "whenever oxidation is initiated on a given fatty acid molecule it proceeds practically instantaneously at alternate carbons along the entire chain to complete disruption into ... two carbon units which are at once reassembled into ketones." This hypothesis sponsored by Stadie then differs from that proposed originally by MacKay by utilizing the theory of multiple alternate oxidation as the mechanism of formation of a 2-carbon compound rather than the theory of successive β oxidation. According to the theory of successive β oxidation, cleavage of the carbon to carbon bond at the β -carbon atom must precede the oxidation at the next β -carbon atom. It would thus be expected that when the 2-carbon units are reassembled into ketone bodies the C^{13} concentration of the carbonyl and carboxyl carbons of acetoacetate derived from carboxyl isotopic fatty acids would be equal. Medes, Weinhouse, and Floyd (12) have demonstrated that when carboxyl-labeled butyric acid is oxidized to ketone bodies in liver tissue there is a greater concentration of C^{13} in the carboxyl carbon than in the carbonyl carbon of acetoacetate. They have calculated from their data that 30 per cent of the butyric acid converted to acetoacetate did not undergo fission into a 2-carbon intermediate but was oxidized directly. Since acetoacetate itself does not undergo splitting in liver tissue, it seems quite possible that a 4-carbon intermediate of butyrate metabolism exists which may be either split into 2-carbon fragments or directly converted into the inactive acetoacetate. The fission of the 4-carbon chain of butyric acid is thus not a compulsory step in its conversion to acetoacetate but may result as a side reaction of some intermediate of butyrate oxidation. Although no direct evidence is available for such an assumption, it is also possible that in the oxidation of longer chain fatty acids fission of the

carbon to carbon linkage at the β -carbon may not be a compulsory step before further activation, oxidation, and cleavage of the carbon to carbon bond at other places along the molecule occur. Evidence presented in this paper might favor this possibility. As previously mentioned, Weinhouse *et al.* obtained equal concentrations of C^{18} in the carboxyl and carbonyl carbons of acetoacetate formed from carboxyl isotopic octanoate in liver slices. In our own experiments with octanoate and liver slices, however, the acetoacetate isolated at the conclusion of the experiment contained a significantly greater concentration of C^{18} in the carboxyl carbon than in the carbonyl carbon. From the data in Table II, it may be calculated² that 20 per cent of the final acetoacetate contained isotope in the carboxyl position only, whereas 80 per cent contained isotope in both carboxyl and carbonyl positions. This might indicate that 80 per cent of the newly formed acetoacetate arose by reactions which involved a 2-carbon compound and that 20 per cent did not. Although these experiments are admittedly neither direct nor conclusive evidence that multiple alternate oxidation exists, they might receive that interpretation.

It would thus seem from experiments reported to date that the formation of a 2-carbon intermediate plays an important rôle in fatty acid catabolism to acetoacetate. The initial reactions involved in the further breakdown of acetoacetate and the exact mechanism of disintegration of fatty acids into acetoacetate are, however, open to further investigation.

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SUMMARY

A study of the metabolism of fatty acids and ketone bodies in liver tissue has been made with the use of carboxyl isotopic octanoic acid and acetoacetate labeled either in the carbonyl or carboxyl position. It has been found that acetoacetate is not split to a measurable extent into 2-carbon fragments which recondense randomly into acetoacetate again. The results of the experiments with isotopic acetoacetate differ in some respects from previous experiments of others concerning the oxidation of carboxyl isotopic octanoate to acetoacetate. From the experiments reported in the present paper the conclusion is reached that the appearance of isotope in the carbonyl carbon of acetoacetate during the metabolism of carboxyl isotopic octanoate in liver slices does not result from a pre-

² The method of making the above calculation and the assumptions involved therein may be found in a paper by Medes *et al.* (12).

liminary formation of carboxyl isotopic acetoacetate and a secondary splitting of this isotopic species into 2-carbon fragments which recondense randomly to form acetoacetate labeled in both carbonyl and carboxyl positions.

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A SYNTHESIS OF METHIONINE CONTAINING RADIO-CARBON IN THE METHYL GROUP

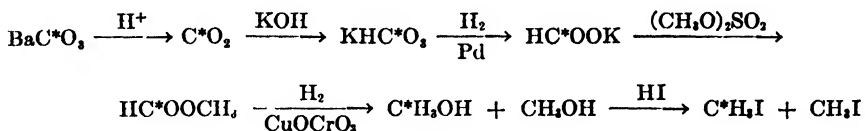
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In connection with investigations under way in this laboratory concerning the metabolism of compounds containing methyl groups, it was desired to prepare methionine in which the methyl group was labeled with C¹⁴. Methionine labeled with deuterium in the methyl group has been used by du Vigneaud and coworkers (1, 2) in studies of transmethylation. The extremely sensitive methods available for the detection of radioisotopes indicated that methionine containing the methyl group labeled with radiocarbon might make possible an amplification and extension of the results obtained with the deuterio compound.

The synthesis of the desired compound involves essentially a satisfactory synthesis of the correspondingly labeled methyl iodide, since methionine can be conveniently prepared by the condensation of methyl iodide with the sodium derivative of homocysteine in liquid ammonia (3). The synthesis of methyl iodide from the radioactive barium carbonate available as starting material was accomplished by the following series of reactions.



Yields of compounds in each of the above steps were in the range, 90 to 100 per cent. The over-all yield of radiocarbon, from barium carbonate to methionine, was 50 per cent. The 2-fold dilution of radioactivity inherent in the method was not considered disadvantageous in the present work, since a 10-fold dilution of the original radioactivity was desired in the final product. The conversion of barium carbonate to potassium formate was carried out without dilution of the radioactive barium carbonate, on a 0.77 mm scale. The radioactive potassium formate was then diluted 5-fold with ordinary potassium formate and the diluted material was carried through the remaining steps.

It is obvious that the radioactive intermediates produced in this series

of reactions are of value in syntheses of other compounds containing isotopic carbon.

A series of reactions similar to those shown above has been used by Harman, Stewart, and Ruben (4) for the incorporation of short lived radio-carbon C^{14} into methyl iodide. In the procedure used by these authors, isotopic carbon dioxide was reduced to formate enzymatically by means of *Bacterium coli*. The formic acid was esterified by treatment with methyl alcohol and sulfuric acid, and then purified by fractional distillation. Yields in the carbon dioxide reduction step were not reported.

Direct catalytic reduction of carbon dioxide to methanol at approximately 400 atmospheres of pressure has been reported (5), but the applicability of the method to small scale work was not investigated by us because equipment for lower pressures was more readily accessible.¹

In the work reported herein, carbon dioxide was liberated from the sample of radioactive barium carbonate by treatment with perchloric acid (6), and was then absorbed by an amount of potassium hydroxide solution sufficient to provide a slight excess over that required to form potassium bicarbonate. The small amount of unabsorbed carbon dioxide was recovered as barium carbonate. The bicarbonate solution, adjusted to the correct pH, was reduced by the method of Bredig and Carter (7). These authors obtained a 75 per cent yield of formate by the reduction of potassium bicarbonate at 60 atmospheres of hydrogen pressure. We found that at a pressure of approximately 100 atmospheres a sample of potassium bicarbonate yielded 98 to 99 per cent of the theoretical amount of formic acid. Formic acid was determined by the reduction of mercuric chloride to mercurous chloride (8). In an experiment in which non-isotopic barium carbonate was converted to potassium bicarbonate and then to potassium formate, the over-all yield of formate determined by the above method was 94 per cent.

Direct esterification of potassium formate was carried out with methyl sulfate by the method of Graebe (9). This procedure has been used to obtain practically quantitative yields in the esterification of isotopic potassium acetate (6). It appeared to work equally well in the preparation of methyl formate. In a preliminary experiment, a sample of methyl formate so prepared from non-isotopic potassium formate possessed a refractive index ($n_D^{4.5} = 1.3508$) identical with that obtained for a sample of redistilled methyl formate.

¹ A synthesis of methyl iodide containing C^{14} , involving the direct reduction of carbon dioxide to methanol at high pressures, has been recently reported (Tolbert, B. M., in preliminary abstracts of a symposium on isotopes sponsored by the Committee on Growth of the National Research Council, University of Chicago, March 3 and 4, 1947; see also *J. Am. Chem. Soc.*, **69**, 1529 (1947)).

The reduction of methyl formate to methanol was carried out at atmospheric pressure with copper chromite catalyst, as described by Harman, Stewart, and Ruben (4). When a sample of ordinary methyl formate was carried through this procedure, practically complete reduction to methanol occurred, as evidenced by a refractive index of $n_D^{20} = 1.3292$ for the condensed vapors from the reduction reaction, compared with $n_D^{20} = 1.3288$ observed for a purified sample of methanol.

In the reduction of the methyl formate labeled with radiocarbon the methanol was not collected; the vapors were instead passed directly into boiling hydriodic acid, as described by Harman, Stewart, and Ruben. The methyl iodide vapors from this step were purified, dried, and condensed. In a preliminary experiment, a sample of non-isotopic potassium formate was converted by the above procedure to methyl iodide with a refractive index ($n_D^{46} = 1.5421$) identical with that of a sample of purified methyl iodide. In another experiment the over-all yield of methyl iodide from 326 mg. of potassium formate was determined by conversion of the methyl iodide to its solid derivative, tetramethylammonium iodide, and was found to be 83 per cent.

Ordinarily, in the conversion of methyl iodide to methionine by condensation with the sodium derivative of homocysteine in liquid ammonia, a slight excess of methyl iodide is used. To minimize losses of radiocarbon in this step, it was considered advisable to use a slight excess of benzylhomocysteine. However, difficulty was experienced in obtaining pure methionine by this procedure. The reaction was therefore carried out by the use of a slight excess of benzylhomocysteine, the addition of the radioactive methyl iodide to the reduced compound, and then the addition of a small amount of non-isotopic methyl iodide to react with the excess homocysteine. The radioactive L-methionine obtained by this procedure was analytically and optically pure after one crystallization.

EXPERIMENTAL

All reactions involving radioactive gases or volatile liquids were carried out in a hood.

Potassium Bicarbonate—151.7 mg. of barium carbonate, containing approximately 0.94 millicurie of C^{14} (purchased from the Clinton Laboratories of the Monsanto Chemical Company), were placed in the reaction chamber (4) of the apparatus illustrated in Fig. 1. The separatory funnel contained 10 ml. of 40 per cent perchloric acid (6). Inside trap *B* was placed a glass test-tube, 18 × 180 mm., the side of which carried a small vent-hole 60 to 70 mm. from the bottom. In this tube, which was used later as the glass liner for the hydrogenation bomb, was placed 1.0 ml. of 0.832 *N* potassium hydroxide solution. The trap and contents were cooled in a dry

ice-cellosolve mixture, and the apparatus was flushed out with a stream of carbon dioxide-free nitrogen gas. The alkali trap was then cooled in liquid nitrogen, and the perchloric acid solution was added dropwise to the barium carbonate, with a slow stream of nitrogen passing through the apparatus. After 45 minutes the stop-cock connecting the trap and separatory funnel was closed, the trap was evacuated by means of an oil pump to 1 or 2 mm. pressure, and the second stop-cock was closed. The evacuated trap was allowed to stand at room temperature for 24 hours.

The contents of the trap were refrozen by means of a dry ice-cellosolve mixture, and a small amount of carbon dioxide gas remaining in the trap was flushed out by means of a stream of nitrogen gas, and collected in a scrubber containing barium hydroxide. To the frozen solution in the trap

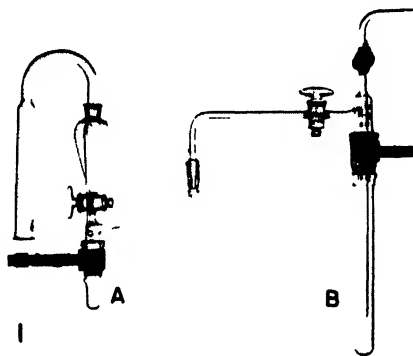


FIG. 1. Apparatus for the conversion of barium carbonate to potassium bicarbonate; one-eighth actual size.

were added 2 to 3 ml. of an aqueous suspension containing 50 to 100 mg. of freshly prepared palladium black catalyst, and this mixture was allowed to freeze as a layer above the carbonate-bicarbonate solution. The test-tube containing the frozen mixture was removed from the trap and sealed off at the top. The liquid was allowed to melt; a drop of phenolphthalein solution was added through the side vent-hole, and 0.1 *N* sulfuric acid was added until the solution was only faintly pink.

Potassium Formate—The tube containing the potassium bicarbonate and palladium black catalyst was placed in an Aminco high pressure hydrogenation bomb. The apparatus was filled with hydrogen at tank pressure (approximately 100 atmospheres) and heated at 70° with shaking for 24 hours. The hydrogenation mixture was then filtered to remove the catalyst, the filtrate being collected in flask *C* of the apparatus illustrated in Fig. 2. To this solution were added 258 mg. of non-isotopic potassium

formate, and the solution was evaporated to a semisolid mass in a stream of hot, dry air. The material was dried further *in vacuo* over phosphorus pentoxide and powdered by means of a glass rod.

Methyl Formate—To the dry, powdered potassium formate was added 0.6 ml. of freshly distilled methyl sulfate in such a manner as to wet the ground glass joint of the flask, which was then attached to the apparatus, as shown in Fig. 2. The reaction flask was heated slowly over a period of 2 hours to a temperature of 185° , by means of an oil bath, with a slow stream of nitrogen passing through the apparatus. The methyl formate was condensed in trap *D*, which was cooled in a dry ice-cellosolve mixture. The uncondensed gases were passed through a barium hydroxide scrubber. The volume of methyl formate collected in the bottom of the trap was approximately 0.2 ml.

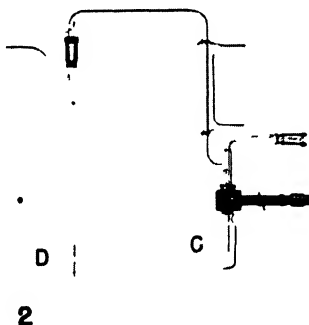


FIG. 2. Apparatus for the esterification of potassium formate; one-eighth actual size.

Methyl Iodide—The apparatus illustrated in Fig. 3 was used for the reduction of the methyl formate to methanol and conversion of the methanol to methyl iodide. Tube *E* contained copper chromite catalyst supported on silica chips, prepared according to the directions of Harman, Stewart, and Ruben (1). Flask *F* contained 10 ml. of freshly distilled, constant boiling hydriodic acid, heated under a reflux by means of an oil bath held at 135° . In scrubber *G* were placed 4 ml. of an aqueous suspension of red phosphorus, and tube *H* contained calcium chloride. Trap *J*, in which the methyl iodide was collected, was cooled by a dry ice-cellosolve mixture. The tube containing the catalyst (*E*) was heated by means of an electric furnace and was held at 160° during the reduction.

The trap (*D*) containing the methyl formate was removed from its dry ice-cellosolve bath and attached to the reduction tube (*E*), as shown in Fig. 3. A slow stream of hydrogen was passed through the train. The methyl formate, which was allowed to warm to room temperature, slowly

evaporated in the stream of hydrogen and was carried into the catalyst chamber. The methyl formate evaporated completely within an hour, after an additional 30 minutes trap *J* containing the methyl iodide was disconnected. The volume of methyl iodide in the trap was approximately 0.37 ml.

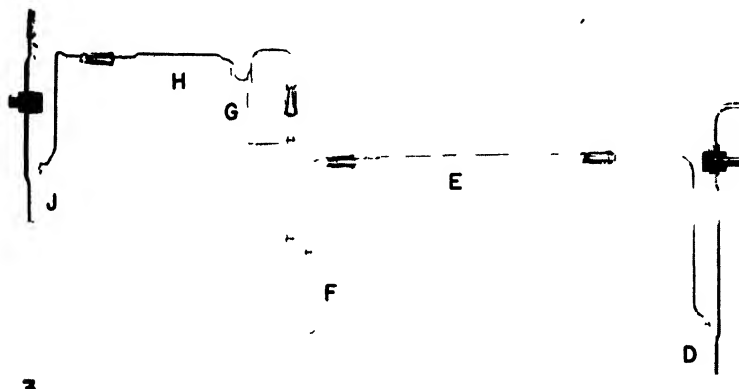


FIG. 3. Apparatus for the conversion of methyl formate to methyl iodide; one eighth actual size.

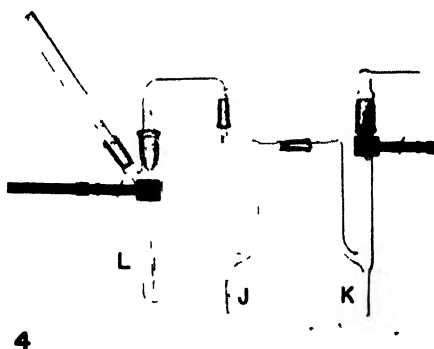


FIG. 4. Apparatus for the preparation of methionine from methyl iodide; one-eighth actual size.

L-Methionine The conversion of methyl iodide to methionine was carried out in the apparatus illustrated in Fig. 4. In flask *L* were placed 1.55 gm. of dry, powdered *S*-benzyl-*L*-homocysteine (10). Dry liquid ammonia (30 ml.) was distilled into the flask, which was cooled in a dry ice-cellosolve mixture. Dry nitrogen was bubbled through the flask to effect more rapid dissolving of the solid. Sodium wire was added to the solution

in small amounts until the blue color due to excess sodium remained for several minutes after the last addition. Then the temperature of the bath surrounding the reaction flask was raised to -50° , the trap (*J*) containing the methyl iodide was attached, and a slow stream of nitrogen was passed through the apparatus. Trap *K* was cooled in a dry ice-cellosolve mixture to remove traces of moisture from the nitrogen used to sweep the methyl iodide in trap *J* into the reaction flask *L*. The trap containing the methyl iodide was warmed to 50° . All the methyl iodide had evaporated within 30 minutes. A nitroprusside test on a small sample of the reaction mixture was positive; so 0.025 ml. of non-isotopic methyl iodide was added to the reaction flask through the side arm. A second 0.025 ml. addition was necessary before the nitroprusside test became negative. The liquid ammonia was then allowed to evaporate slowly, with a stream of nitrogen passing through the solution.

The white, solid residue was dissolved in 15 to 20 ml. of water. Hydriodic acid was added until the solution was acid to litmus but still alkaline to Congo red. Insoluble material was separated by filtration. The filtrate was concentrated *in vacuo* to 5 or 10 ml. and then heated to dissolve the crystalline material which had separated. To the hot solution 100 ml. of boiling absolute ethanol were added. The mixture was kept at 5° overnight. The silvery crystals of L-methionine were collected by filtration, washed with alcohol and ether, and dried. The yield was 860 mg. The material melted with decomposition at 283° , a value identical with that observed for a pure sample of L-methionine. The optical rotation was $[\alpha]_D^{22} = -7.75^{\circ}$ (2 dm. tube, $c = 0.95$, in water).

$C_5H_{11}O_2NS$.	Calculated.*	N 9.38,	S 21.48
(149.2)	Found.	" 9.32,	" 21.70

Radioactivity Measurements—In order to determine the over-all yield of radioactivity in the conversion of radioactive barium carbonate to methionine, samples of both the starting material and the final product were converted to carbon dioxide by appropriate methods. The carbon dioxide was absorbed in sodium hydroxide solutions, which were then made up to definite volumes. To aliquots of these solutions was added enough sodium carbonate solution to yield about 50 mg. of barium carbonate when the solutions were treated with barium chloride. The precipitation was carried out in hot solution in order to yield a more coarsely crystalline barium carbonate. The precipitates were filtered and collected by the method described by Henriques *et al.* (11) for the precipitation and collection of benzidine sulfate. In our method the precipitates covered

* The amount of C^{14} present does not significantly affect the analytical results.

an area of 2.32 sq. cm. on the paper disks, and therefore had thicknesses greater than 20 mg. per sq. cm. The radioactivity measurements were made with the use of a thin mica window bell-shaped Geiger-Müller counter (11) and a scaling circuit. After correction for background and variation in thickness of the samples (12, 13), it was found that 1.89×10^{-3} mg. of the radioactive barium carbonate gave 485 counts per minute, while barium carbonate prepared from 1.93×10^{-3} mg. of the radioactive methionine gave 451 counts per minute. The 151.7 mg. of barium carbonate used as the starting material accordingly represented 3.89×10^7 counts per minute, and the 860 mg. of methionine 2.01×10^7 counts per minute. The over-all yield of radioactivity was therefore approximately 51 per cent.

SUMMARY

A synthesis of L-methionine containing radiocarbon (C^{14}) in the methyl group has been described. Radioactive barium carbonate, the starting material, was converted to potassium bicarbonate, which was reduced in the presence of palladium black catalyst to potassium formate. Esterification of the potassium formate was carried out with methyl sulfate. The methyl formate so obtained was reduced to methanol by means of a copper chromite catalyst. Methyl iodide, prepared by the treatment of the methanol with hydriodic acid, was condensed with the sodium derivative of homocysteine in liquid ammonia to yield methionine.

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BIOCHEMICAL EXPERIMENTS WITH A SYNTHETIC PREPARATION HAVING AN ACTION ANTAGONISTIC TO THAT OF PTEROYLGLUTAMIC ACID

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Deficiencies of pteroylglutamic acid have been produced in various species by feeding purified diets. The signs of the deficiency in chicks were reported to include slow growth, a macrocytic hyperchromic anemia, reduction in leucocytes and thrombocytes (1), and impaired feathering and pigmentation (2). The deficiency in rats was produced by adding sulfonamides to a purified diet. The rats developed a syndrome which was marked by agranulocytosis, leucopenia, hypoplastic bone marrow, anemia, and slow growth, and which responded to pteroylglutamic acid (3).

A synthetic product with "displacing" activity for pteroylglutamic acid was recently described (4) as "methylfolic acid," prepared by allowing 2,4,5-triamino-6-hydroxypyrimidine and *p*-aminobenzoyl-D-glutamic acid to react with 2,3-dibromobutyraldehyde as described by these laboratories (5). The product was found to antagonize the growth-promoting action of pteroylglutamic acid for *Streptococcus faecalis* R.

In the present communication, results are described which had been obtained with a similar product previous to the appearance of the report by Martin and coworkers (4). The crude "antagonist" was prepared by Dr. M. E. Hultquist and Dr. J. M. Smith, Jr., Calco Chemical Division, American Cyanamid Company, by using *p*-aminobenzoyl-L-glutamic acid rather than the D compound in the reaction. The crude product was fed to weanling female albino rats which received a purified diet plus 1 per cent sulfasuxidine. The basal diet contained all the available crystalline B vitamins except pteroylglutamic acid. The preparation was found to accelerate and intensify the signs of pteroylglutamic acid deficiency in rats. The effect of the preparation was apparently completely prevented by addition of suitable amounts of pteroylglutamic acid to the diet.

EXPERIMENTAL

Preparation of Crude Antagonist—The reaction previously described (5) was used, except that α,β -dibromobutyraldehyde was used rather than the corresponding dibromopropionaldehyde. The crude precipitate, hereafter

referred to as the "antagonist," was separated with a filter aid and was dried without attempts at purification.

Microbiological Effect—The antagonist was found to inhibit the growth-promoting effect of pteroylglutamic acid on *Streptococcus faecalis* R and *Lactobacillus casei*. The medium of Landy and Dicken (6) was modified so as to contain the same amino acid, purine, pyrimidine, and vitamin

TABLE I

Effect of Antagonist and Pteroylglutamic Acid (PGA) on Growth of Streptococcus faecalis R and Lactobacillus casei

Organism	PGA per 10 ml. medium	Antagonist per 10 ml. medium	Growth	Apparent minimum ratio of antagonist to PGA for maximum inhibition
<i>S. faecalis</i> R	γ	γ	optical density	
	None		0.02	
	0.0025		0.40	
	0.01		0.88	
	0.01	0.1	0.37	
	0.01	0.3	0.04	30
	0.1	0.8	0.30	
	0.1	1.2	0.12	
	0.1	2.0	0.04	20
	1.0	1.0	1.10	
	1.0	3.0	0.66	
	1.0	10	0.30	
<i>L. casei</i>	1.0	30	0.14	30
			ml 1 N NaOH	
	None		1.1	
	0.0004		4.2	
	0.002		9.4	
	0.001	0.3	4.0	
	0.001	1.0	1.6	1000
	0.001	3.0	0.5	
	0.1	1.0	9.6	

supplements as were used by Teply and Elvehjem (7). The growth periods for *Streptococcus faecalis* R were 16 hours and for *Lactobacillus casei* 70 hours. The inhibition was reversible, and is illustrated by the data in Table I.

Care of Animals—Weanling female albino rats of the Wistar strain were kept in cages with $\frac{1}{2}$ inch mesh wire floors. The basal purified diet (Diet 1) had the following composition: glucose (cerelose) 72 gm., washed casein (Labco) 20 gm., salt mixture (8) 4 gm., corn oil (Mazola) plus vitamins A, D, and E 3 gm., succinylsulfathiazole 1 gm., choline chloride 0.1 gm., inositol 0.1 gm., niacinamide 5 mg., calcium pantothenate 5 mg., thiamine

hydrochloride 1 mg., riboflavin 1 mg., pyridoxine hydrochloride 1 mg., *p*-aminobenzoic acid 1 mg., 1-acetoxy-2-methyl-4-naphthyl sodium phosphate 0.5 mg., biotin 0.02 mg. 3 gm. of the corn oil preparation contained vitamin A (acetate) 1500 U. S. P. units, vitamin D (delsterol) 200 A. O. A. C. chick units, mixed tocopherols 34 mg. Five rats were used in each group.

TABLE II

Effect of Antagonist and Pteroylglutamic Acid (PGA) on Growth of Rats Fed Diet 1

Experiment No	Group No	Supplement		Gain in weight			
		PGA per kilo diet	Antagonist per kilo diet	2 wks.	4 wks.	6 wks.	8 wks.
		mg	gm.	gm.	gm	gm.	gm.
1	1	0	0	44	66	85	
	2	1 0	0	42	71	94	
	3	0	1.0	31	29	*	
	4	0.3	1.0	42	64	72	
	5	3.0	1.0	50	86	104	
	6	30	1 0	49	82	112	
	7	0	10	29	17	*	
	8	1.0	10	33	22	*	
	9	10	10	47	79	94	
	10	100	10	45	74	95	
2	11	0	0	18	45	60	66
	12	1.0	0	25	54	81	87
	13	0	10	17	6	*	*
	14	1.0	10	24	33	†	†
	15	10	10	22	47	65	79
	16	100	10	28	57	83	92

* All animals dead.

† Animals removed for recovery experiment.

Results

Addition of the antagonist to Diet 1 produced a syndrome similar to but more severe than that described elsewhere for rats which received a purified diet plus sulfonamides (3) (Table II). By using various levels of the antagonist and pteroylglutamic acid it was readily possible to demonstrate a competition between the antagonist in producing the syndrome and pteroylglutamic acid in preventing it. After the syndrome had been produced, it was possible to reverse it by increasing the dietary level of pteroylglutamic acid and to study the regenerative changes concerned with recovery.

It was found that, when the level of pteroylglutamic acid added to the diet was in excess of approximately 0.3 mg. per gm. of antagonist, the rats

were protected against pteroylglutamic acid deficiency. If, however, the level of pteroylglutamic acid was 0.3 mg. or less per gm. of antagonist, the animals developed acute signs of pteroylglutamic acid deficiency which became progressively more intense as the ratio of antagonist to pteroylglutamic acid was widened (Table III). The deficiency was characterized by a number of parallel effects, including a slowing of growth, a reduction

TABLE III

Effect of Antagonist and Pteroylglutamic Acid (PGA) on Hematology of Rats Fed Diet 1

Experiment No	Group No.	Supplement		Hemoglobin, gm. per 100 ml.				White blood count, cells per c mm $\times 10^3$				Granulocyte count, cells per c mm.			
		PGA	Antagonist	1 wk.	3 wks	5 wks	7 wks	1 wk	3 wks	5 wks	7 wks	1 wk.	3 wks	5 wks	7 wks
		mg. per kg.	gm. per kg.												
1	1	0	0	14.0	15.1	18.3			8.2	12.6		1500	1500		
	2	1.0	0	14.5	16.6	19.3			10.5	15.2		2400	2600		
	3	0	1.0	14.5	13.6	10.5			2.8	0.8		84	16		
	4	0.3	1.0	14.7	13.1	18.1			7.8	13.3		470	540		
	5	3.0	1.0	15.8	15.1	20.9			10.9	13.7		1500	1100		
	6	30	1.0	14.9	15.6	21.3			11.3	14.9		1800	1500		
	7	0	10	15.4	10.5	*			0.8	*		8	*		
	8	1.0	10	15.4	12.2	*			2.5	*		100	*		
	9	10	10	13.8	15.1	20.2			9.5	14.2		1200	1100		
	10	100	10	16.0	15.7	20.6			15.5	18.2		2900	1600		
2	11	0	0	13.2	12.1	15.2	16.5	5.6	9.7	7.0	5.5	1200	1500	630	550
	12	1.0	0	17.0	13.1	15.9	17.3	7.9	14.8	12.8	14.1	990	2700	2900	4000
	13	0	10	13.8	10.6	*	*	5.7	2.2	*	*	970	66	*	*
	14	1.0	10	14.7	13.6	12.2	†	4.5	4.7	3.8	†	670	140	110	†
	15	10	10	15.0	13.7	17.1	17.8	9.4	9.5	6.1	10.0	2100	1400	1200	1400
	16	100	10	11.7	15.8	17.6	18.3	14.4	9.8	12.0	13.8	3100	1400	2200	2300

* All animals dead.

† Animals removed for recovery experiment.

of the per cent hemoglobin, a similar but more marked reduction of the white cell count, and, in the differential white count, a greater reduction in the granulocytes than in the lymphocytes. The first change observed was a loss in weight. During this period, inanition developed and the fur became very rough and unkempt in appearance. Encrustation of the vibrissae with a red pigment (9) was observed, together with severe diarrhea. Most of the animals found difficulty in eating and appeared to have a tenderness of the mouth. There was always an excellent correlation between the blood morphology, weight changes, and the external signs of

the condition. The animals were usually in a moribund condition within 1 to 2 weeks after the onset of the syndrome. At this time the rats were in a state of extreme emaciation, a general cytopenia had developed, and loss of body weight was about 20 per cent.

The most significant observation at autopsy was the condition of the oral cavity. All animals which died from the syndrome produced by the antagonist were found to have necrotic and ulcerative changes in the oral cavity. Many rats developed deep ulcers on the lip. There was usually a general necrotic condition within the mouth. A severe gingivitis was common and many animals developed deep ulcerations at the margins of the gums. In several animals ulcerations were seen on the central parts of the anterior surface of the tongue. In a few rats deep abscesses in the sinuses were found associated with the erosion of the palate.

The lungs were usually inflamed, with considerable congestion. The gastrointestinal tract of most animals was empty and atonic. Only the genital system was found to be altered. The uteri were always small and atrophic. The liver, spleen, heart, and other organs were normal in size and appearance.

Bone Marrow—The distribution and morphology of the cells in the bone marrow were in accord with the hematological changes found in the peripheral blood. All marrow samples were normal in appearance and no fatty infiltration was apparent. Samples were obtained at autopsy from the femur and smears were prepared by the touch technique with Wright-Giemsa stain. Two slides were prepared from each animal; the values are given in Table IV as cells per 100 nucleated cells after counting 1000 cells on each slide.

Animals 12-2 and 12-3 received an adequate pteroylglutamic acid supplement of 1.0 mg. per kilo of diet and were used as the controls. The values for Animals 12-2 and 12-3, therefore, indicate the normal ranges for the various types of nucleated cells of the marrow. The other rats received supplements per kilo of Diet 1 as follows: Animal 3-4, 1.0 gm. of antagonist; Animal 13-3, 10 gm. of antagonist; Animals 6-1, 6-3, and 6-4, 30 mg. of pteroylglutamic acid plus 1.0 gm. of antagonist; Animals 14-4, 14-1, 14-2, and 14-5, 1.0 mg. of pteroylglutamic acid plus 10 gm. of antagonist until the 5th week, following which the pteroylglutamic acid level was increased to 100 mg. The morphology of the marrow from a rat with a severe antagonist-induced syndrome is indicated by the results obtained with Animals 13-3 and 3-4. The data show that the proportion of nucleated erythroid cells was increased. Furthermore, although deeply staining blast cells of this group were seldom seen in the controls, a considerable number were found in the erythroid series of the animals with the syndrome. In the

myeloid series very few mature granulocytes were found. These data indicate that the antagonist exerted a very pronounced effect on the cyto-

TABLE IV

Effect of Antagonist on Cytology of Bone Marrow of Rats As Compared with Rats Receiving Protective and Curative Levels of Pteroylglutamic Acid

Animal No	Condition	Total wks on experiment	Wks on recovery diet	Marrow count			
				Nucleated erythroids	Mature granulocytes including metamyelocytes	Other nucleated cells*	Doubtful cells
				per cent	per cent	per cent	per cent
12-2	Normal	10		30-32	17-18	45-48	2-4
12-3	"	10		31-33	30-32	30-32	3-4
13-3†	Deficient	5		40-42	0	51-53	4-5
3-4‡	"	4		40-41	0-1	55-56	3-4
6-1	Protected	5		28-30	37-38	28-29	4-5
6-3	"	10		29-30	36-37	30-31	3-4
6-4	"	10		27-29	34-36	35-36	1-2
14-4§	Recovery	6	1	21-23	29-32	43-44	4-5
14-1		9	4	12-13	52-55	25-27	6-9
14-2¶	"	9	4	28-37	31-41	27-28	3-4
14-5¶	"	9	4	30-34	28-33	27-30	3-5

* Majority stem cells

† Animal 13-3 had an aregenerative marrow. The increased proportion of cells of the nucleated erythroid group and the appearance of a considerable number of deeply stained blast cells of this series resulted in a morphological picture similar to megaloblastic arrest. The marrow, furthermore, showed a complete suppression of the maturation of granulocytes.

‡ The bone marrow for Animal 3-4 was similar to that for Animal 13-3. Mitotic figures were rare. Normoblasts and erythroblasts were seldom found and the erythroid group consisted primarily of deeply stained blast cells.

§ The marrow showed regeneration of cells of the myeloid series, although the cells were predominantly metamyelocytes rather than mature granulocytes. Within the erythroid group the number of normoblasts was low and the marrow was considered aregenerative for this series.

|| There was a hyperregeneration of the myeloid series, as indicated by a large number of granulocytes (mostly metamyelocytes). The marked scarcity of normoblast, erythroblasts, and erythrocytes, as well as the presence of megaloblasts, indicated that there was an aregeneration of the marrow in the erythroid series.

¶ The marrow of these two animals appeared to be normal, indicating that recovery had taken place.

poietic mechanism of the bone marrow. The maturation of cells of the erythroid series and the production of mature granulocytes were seriously impaired. Group 6 received the same dietary level of antagonist as the rats of Group 3; however, sufficient pteroylglutamic acid was added to

overcome the effect of the antagonist. The values for these animals were in good agreement with those obtained for the normal control group. The present findings with bone marrow are similar to the results previously reported by Endicott and coworkers (10) for rats with pteroylglutamic acid deficiency induced by feeding a purified diet plus succinylsulfathiazole.

The experimental reversal of the deficiency syndrome in rats is described below. Observations were made on the marrow changes of these animals. The animals of Group 14 developed the syndrome after receiving the antagonist; then the pteroylglutamic acid level was increased and most animals recovered from the severe cachexia associated with the deficiency.

Animals 14-2 and 14-5 recovered completely in growth and appearance. When the animals were sacrificed 4 weeks after the reversal was begun, the marrow was normal. Animal 14-4 was sacrificed 1 week after the pteroylglutamic acid level was increased and the data indicate that only a partial recovery of the normal cytopoietic function of the bone marrow had taken place. Animal 14-1 remained refractory to the therapy and failed to show significant gains in body weight. This animal displayed the most severe deficiency symptoms previous to the attempted reversal and died as the experiment was being terminated. The aberrant morphology of the marrow from this animal is evident from Table IV.

Reversal of Effect of Antagonist—The results are shown in Fig. 1. The rats from Group 14 were used, which had received a supplement of 10 gm. of antagonist and 1 mg. of pteroylglutamic acid per kilo of diet. The "reversal" was attempted just prior to the expected appearance of the terminal moribund state. At the end of the 5th week all of the animals were still alive but they were losing weight, a severe cytopenia was found, and all had the characteristic chromodacryorrhea. At this time the pteroylglutamic acid supplementation was increased to 100 mg. per kilo of diet. One animal died on the 1st day but the remaining rats quickly showed signs of recovery. The ruffled fur and "bloody tears" disappeared in about 1 week. The animals began to gain weight, the hemoglobin was restored to the normal level within 3 weeks, and there was a striking leucopoiesis, the white cell count increasing from about 4000 to 57,000 cells per c.mm. within 1 week. The augmentation was primarily in the granulocyte series. Only one animal failed to respond. The remaining rats recovered rapidly and displayed normal appearance, growth rates, and blood morphology within a few weeks.

The animals were sacrificed 4 weeks after the change and no oral lesions were found. The bone marrow at this time was normal in regard to cells of the erythroid and granulocyte series. The only unusual finding was the condition of the spleen. One animal was sacrificed 1 week after the reversal

and the spleen was about 4 times the normal size. This splenomegaly was also found in animals sacrificed at the later time, although the degree of hypertrophy was not as marked.

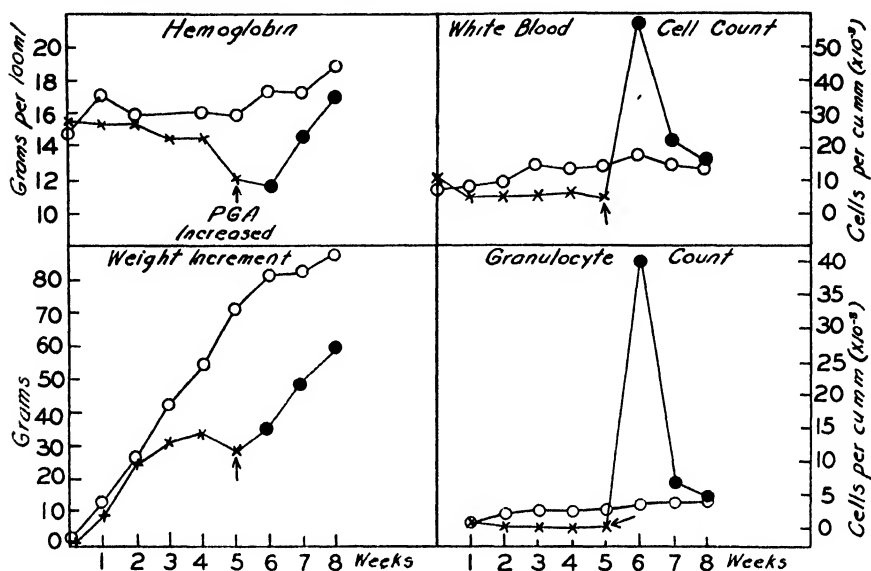


FIG. 1. Graphic representation of changes in hemoglobin, body weight, white blood cell count, and granulocyte count in five rats (Group 12) receiving Diet 1 plus 1 mg. of pteroylglutamic acid per kilo of diet (○) and in three rats (Group 14) receiving Diet 1 plus 10 gm. of antagonist and 1 mg. of pteroylglutamic acid per kilo of diet (×), which was changed (arrows) to Diet 1 plus 10 gm. of antagonist and 100 mg. of pteroylglutamic acid per kilo of diet (●).

DISCUSSION

Differing "inhibition ratios" of the antagonist of pteroylglutamic acid were found for various organisms. The ratio for *Streptococcus faecalis* R was 20:1, for *Lactobacillus casei* about 1000:1, and for rats about 3000:1. The syndrome induced by the antagonist appeared similar to the pteroylglutamic acid deficiency produced in rats on a purified diet plus succinyl-sulfathiazole (3, 10). However, the syndrome produced by the antagonist was much more severe. The results of the present investigations showed that the syndrome may be prevented or reversed by pteroylglutamic acid. The oral lesions noted in this investigation have not been described previously in rats for the pteroylglutamic acid deficiency and recall the lesions described in monkeys (11).

It was stated by Sabin (12) that one of the major problems of modern hematology is to find substances which exert specific effects on the maturation

tion of the erythroid and myeloid cells of the bone marrow. Stimulation or depression of these cytopoietic mechanisms may now be accomplished by the use of pteroylglutamic acid or the antagonist.

Several clinical syndromes are marked by an increase in the count of the cellular elements of the blood. It has been demonstrated in this study that in normal animals a cytopenia may be produced by the antagonist. This preparation might be used experimentally in an attempt to modify blood dyscrasias marked by erythrocytosis or leucocytosis.

Chemical identification of the antagonist was not made, although the product described elsewhere (4) has been designated "7-methylfolic acid."

SUMMARY

1. A crude preparation made by condensing 2,4,5-triamino-6-hydroxypyrimidine and *p*-aminobenzoyl-L-glutamic acid with 2,3-dibromobutyraldehyde was found to antagonize reversibly the effect of pteroylglutamic acid on *Streptococcus faecalis* R, *Lactobacillus casei*, and rats.

2. The syndrome produced in rats by the antagonist appeared similar to but more acute than that described for pteroylglutamic acid deficiency as induced by feeding a purified diet plus succinylsulfathiazole, and was accompanied by oral lesions.

3. Reversal of the syndrome was accompanied by temporarily overcompensatory increases in total white blood cell and granulocyte count. An enlargement of the spleen was noted following recovery.

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A STUDY OF WHEY PROTEINS FROM THE MILK OF VARIOUS ANIMALS*

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The proteins of the plasma and sera of various animals as studied by the electrophoretic technique show extreme species variation but are characteristic and reproducible for a given species (1, 2). It appeared desirable to determine whether the milk proteins of various mammals would show similar marked variation in electrophoretic patterns and whether any marked relation between the proteins of milk sera and blood plasma exists. An electrophoretic and ultracentrifugal study of the milk serum proteins of different animals at various times post partum has revealed characteristic deviations in these proteins, but few if any apparent relationships to the homologous plasma proteins were found except for milk samples taken directly post partum.

EXPERIMENTAL

Fat was removed from the various milk samples by centrifugation, following which the skim milk was adjusted to pH 6.2 (± 0.1) by the addition of dilute hydrochloric acid. For reasons to be discussed, the casein was removed by rennin, a small amount of a commercial preparation sufficient to precipitate the casein within 60 minutes at 35° being used. Following removal of the casein by centrifugation and filtration, the whey was dialyzed against cold running tap water for 16 to 20 hours to remove the major part of the lactose. The dialyzed protein was frozen and dried *in vacuo*.

Electrophoretic analyses of these proteins were carried out in a barbiturate-citrate buffer of pH 8.6 and ionic strength of 0.088, in which the sodium citrate contributed 48 per cent of the ionic strength. The samples were dialyzed against several changes of buffer for 30 to 60 hours at 1°. Since most samples showed varying degrees of turbidity, they were filtered through a thin Seitz pad prior to the final dialysis period. The electrophoretic experiments were carried out in a long, single section cell of 11 ml. capacity at a constant potential gradient of approximately 8.5 volts per cm. and at a temperature of 1°. Electrophoretic mobilities were measured by using the center of the initial boundary as the reference point. Com-

* A portion of this material was presented before the Division of Biological Chemistry of the American Chemical Society at Chicago, September 9-13, 1946.

ponents were designated numerically in the order of increasing mobility, since usually they bore no general relationship to the plasma proteins of the homologous species.

Sedimentation analyses were carried out in the high velocity, oil turbine Svedberg ultracentrifuge. The speed of the rotor was 50,400 R.P.M. The positions of the moving boundaries as a function of time were recorded by the cylindrical lens schlieren method in conjunction with a diagonal knife-edge.

Nitrogen analyses were performed on the skim milk and whey samples and the amount of casein was obtained by difference.

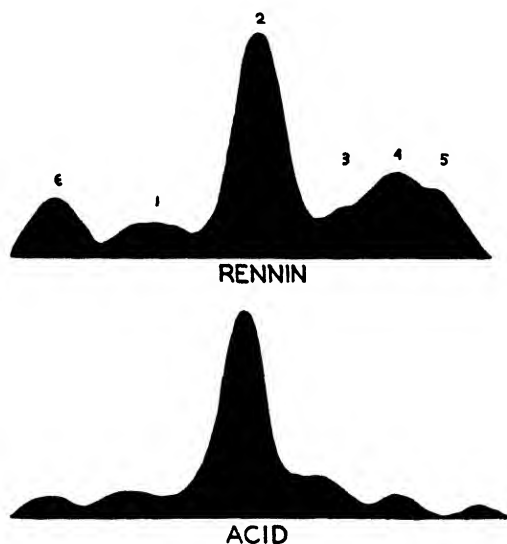


FIG. 1. Comparison of descending electrophoretic boundaries of goat whey after removal of casein by isoelectric precipitation and by rennin.

Results

No differences in the electrophoretic composition of cow whey proteins were observed when the casein was removed by isoelectric precipitation or by treatment with rennin. However, in the case of pooled goat milk, a considerable part of the whey proteins of lower isoelectric point was removed by the acid precipitation. It was found necessary to lower the pH to 4.3 to 4.4 to remove effectively goat casein from these proteins, a pH somewhat lower than that required in the case of the bovine milk. The electrophoretic patterns of goat wheys from the same milk from which casein was removed by rennin and by acid treatment are shown in Fig. 1.

Considerable amounts of the goat whey proteins of the lower isoelectric point were removed by acid precipitation and, since a similar effect might occur with milk from other species, all casein removals were performed with rennin. In the case of human milk, rennin treatment followed by adjust-

TABLE I
Distribution of Nitrogen in Fractions from Milk of Several Animals at Various Times Post Partum

Animal	Days after parturition	Nitrogen		
		Skim milk	Whey	Casein
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Cow	0	2.32	1.58	0.74
	1	1.05	0.51	0.54
	2	0.57	0.13	0.43
	Aged*	0.47	0.12	0.35
Goat	0	1.76	1.22	0.54
	2	0.73	0.28	0.45
	4	0.68	0.22	0.46
	Aged*	0.63	0.17	0.46
Pig	0†	2.50	1.74	0.76
	0‡	2.31	1.81	0.50
	2‡	2.00	1.66	0.34
	3‡	0.60	0.32	0.28
	5‡	0.99	0.56	0.43
	9‡	0.65	0.39	0.26
	13‡	1.04	0.73	0.31
Human	1-2*	0.29	0.25§	0.04
	6-9*	0.28	0.17	0.11
	14*	0.20	0.15	0.05
	40	0.20	0.13	0.07
	90	0.23	0.13	0.10
Sheep	7	1.61	0.54	1.07
	35	0.78	0.20	0.58
Horse	11	0.80	0.40	0.40
	90	0.40	0.21	0.19

* Pooled samples; remainder individual.

† Poland-China sow.

‡ Chester white sow.

§ 31.3 per cent of whey nitrogen dialyzable.

ment of the pH to 4.8 was found necessary to precipitate the casein. The first postpartum milk of swine also offered some difficulties in this respect.

To determine whether appreciable amounts of casein remained with the whey proteins, a sample of casein was prepared by acid precipitation of bovine skim milk and electrophoretic analyses were carried out. In

TABLE II
Electrophoretic and Ultracentrifugal Analysis of Whey Proteins

Species	Days post partum	Per cent and electrophoretic mobility,* constituent No.								Per cent sedimentation constituents†				
		1	2	3	4	5	6	7	8	s ¹⁻²	s ³	s ⁴	s ¹²	s ²⁰
Bovine	0	69.5	5.1	10.3	12.7	2.4				22	68			10
	1	55.7	8.2	17.2	16.9	2.0								
	2	26.3	14.5	22.5	34.6	2.1				75	25			
	Aged‡	10.2	18.0	20.2	48.3	3.3				93	7			
Goat	u*	2.2	3.9	5.0	6.1	7.3								
	0	52.8	31.6	5.2	4.0	6.4				39	59			2
	2	11.0	63.0	9.2	9.6	7.1				89	8			3
	4	9.2	64.8	9.3	10.1	6.5								
Pig (Chester white)	Aged‡	9.5	53.7	8.6	15.9	12.3								
	u	1.7	3.9	5.6	6.5	7.3								
	0		39.6	9.3	11.2	12.5	7.1	20.3						
	3		32.5	20.6	9.8	14.2	7.5	15.4						
Pig (Poland-China)	6		7.4	29.0	16.8	8.4	7.9	30.5						
	14	7.8	4.7	18.4	15.3	15.6	6.6	31.6						
	u	0.8	1.6	3.6	3.5	4.3	5.2	6.4						
	0		45.2	13.9	8.1	6.3	10.4	16.1		38	62			
Human	4		9.0	25.6	17.5	6.2	13.2	29.5						
	9	1.6	11.7	26.3	17.4	5.2	13.3	24.5		87	6	7		
	u	0.9	1.8	2.7	3.7	4.5	5.7	6.5						
	1-2‡	33.6	23.5	30.0	6.0	2.8	4.1			75	10	3	12	
Sheep	6-9‡	35.0	16.5	43.0	1.9	1.0	2.6							
	14	28.7	18.0	40.5	3.5	2.0	7.3							
	40	26.9	5.8	45.3	5.3	6.1	10.6							
	90	25.7	11.9	47.8	4.9		9.7			80	15	3	2	
Horse	u	2.4	3.7	4.8	5.7	6.3	7.4							
	7	8.2	6.3	5.7	46.8	11.6	9.4	10.2	1.8	91	9			
	35		5.8	2.6	63.3	10.8	11.8	4.3	1.4	99	1			
	u	1.6	2.2	2.7	3.8	5.1	6.1	6.8	7.5					
Horse	11§	10.7	5.6	19.2	6.7	14.0	6.8	7.0		92	8			
	90	14.0	5.9	18.3	10.7	40.9	4.2	6.0						
	u	0.7	1.9	3.1	3.8	5.6	7.8	9.1						

* Average mobilities (u) in 1×10^{-8} cm.² volt⁻¹ sec.⁻¹. The mobilities are negative.

† Sedimentation velocities (s_{20w}) in 1×10^{-13} cm. sec.⁻¹ unit field⁻¹.

‡ Pooled samples

§ Two animals.

|| Sedimentation constant 7.6

agreement with previous workers (3, 4), two electrophoretic components were observed. Their mobilities did not agree with any of those of the components of the whey, which suggests that the bovine whey samples studied did not contain significant amounts of casein.

Samples of the various milks showed characteristic changes at various times post partum. In general, these changes were characterized by progressive decrease with time post partum in the nitrogen content of the whey and the components of low electrophoretic mobility. The higher molecular weight components showed similar decreases as revealed by sedimentation analysis. Such observations are compatible with numerous data in the

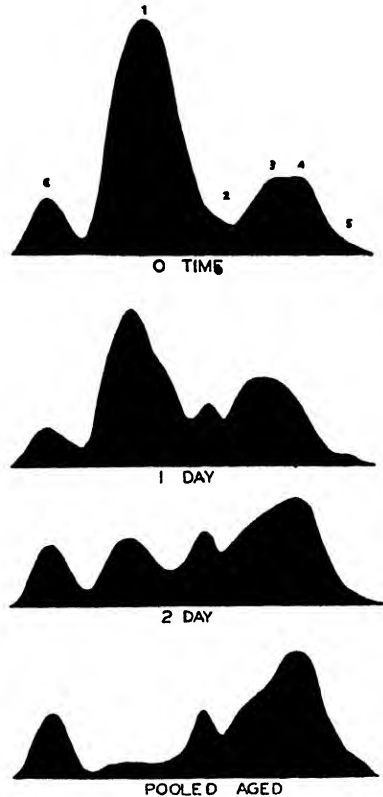


FIG. 2. Descending electrophoretic boundaries of bovine whey at various times post partum.

literature which show marked increases in the albumin-globulin ratio of milk proteins with increase in postpartum time. The amounts of nitrogen found in the three milk fractions of the animals studied at various times following delivery are shown in Table I. The electrophoretic analyses for the same samples are shown in Table II. Numbers by which the components for a given species are designated bear no relationship to the same numbers used with components of other species, but are used merely as a

matter of convenience. Sedimentation analyses of the first and latest postpartum whey samples for each species are also included with the other data in Table II.

A marked change in the concentration of various protein constituents of bovine whey will be apparent from an inspection of Tables I and II. The electrophoretic and sedimentation patterns reflecting these changes are shown in Figs. 2 and 8. In addition to the sedimentation components recorded in Fig. 8, there is present a component with sedimentation con-

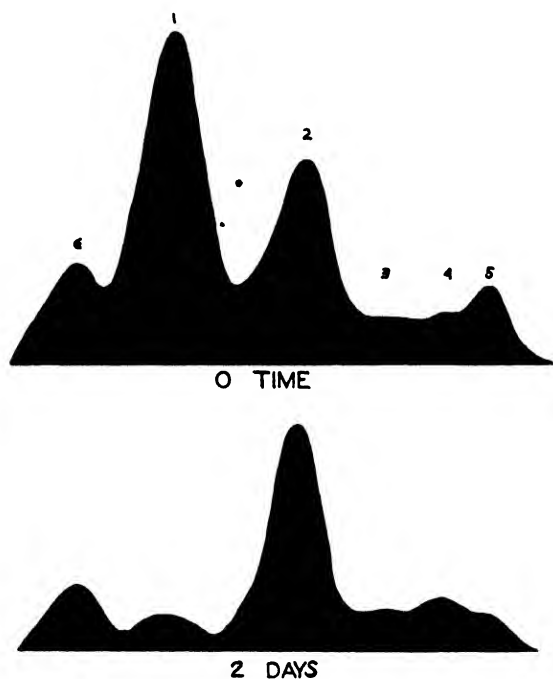


FIG. 3. Descending electrophoretic boundaries of goat whey at 0 and 48 hours post partum.

stant $s_{20w} = 20$ Svedberg units. This component represents about 10 per cent of the total protein. After 2 days, the milk constituents began to approach those of normal pooled milk. The electrophoretic composition of bovine colostrum is analagous to that recorded by Smith (4). The globulins of slower electrophoretic mobility are relatively heterogeneous. They possess a mobility within the range of the γ -globulins separated from bovine serum by Smith (4) and by Hess and Deutsch (5). The electrophoretic pattern of the various bovine milk samples shown in Fig. 2

strongly suggests that several proteins are present in the component designated Component 1.

Relatively rapid changes in the whey proteins of the goat are indicated by the nitrogen analysis. These proteins were essentially normal as indicated by nitrogen and electrophoretic analysis for a sample taken at 48 hours

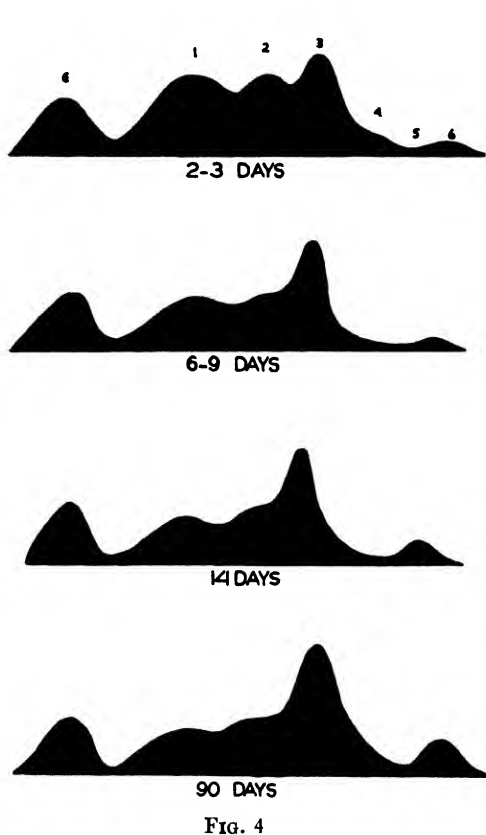


FIG. 4

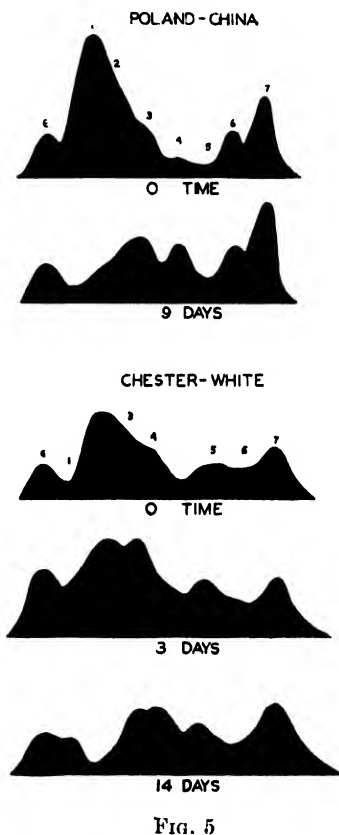


FIG. 5

FIG. 4. Descending electrophoretic boundaries of human whey at various times post partum.

FIG. 5. Descending electrophoretic boundaries of whey of Chester white and Poland-China pigs at various times post partum

post partum (Fig. 3). On the basis of chemical analyses, Bergman and Turner (6) have reported that the goat milk constituents tend to approach a stable condition on about the 3rd to 4th day post partum. The electrophoretic mobility of the fraction of slow movement was close to that reported for the analogous constituent of goat blood plasma (1, 7). More-

over this component was relatively homogeneous in the electrophoretic field. The sedimentation studies (Fig. 8) show changes similar to those recorded for the cow whey.

Relatively slight changes with postpartum time in the electrophoretic and sedimentation diagrams were found in the case of the proteins of human milk whey. However, it can be seen from Fig. 4 that the amount of the electrophoretic Component 1 is largest in samples taken soon after

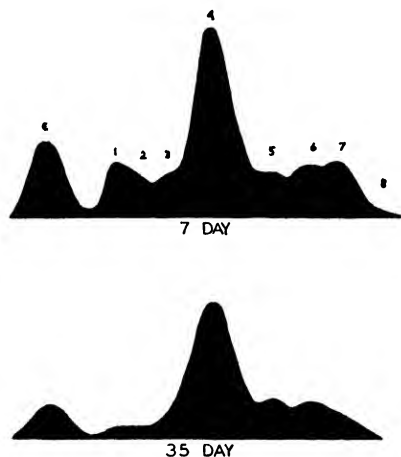


FIG. 6. Descending electrophoretic boundaries of sheep whey at various times post partum.

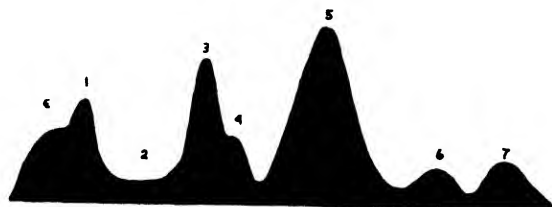


FIG. 7. Descending electrophoretic boundary of horse whey 11 days post partum.

birth. The sedimentation patterns for human milk whey are more complex compared to those observed for the other species; also smaller amounts of the proteins of sedimentation constants $s_{20w} = 1$ to 3 Svedberg units are present.

The electrophoretic patterns of the milk plasma proteins of two breeds of pigs revealed slight differences even within the species, as is shown in Fig. 5. However, postpartum changes similar to those of the other animals studied, as reflected by the electrophoretic and sedimentation analyses

(Fig. 8), are also evident. The whey prepared from milk collected from the Chester white sow 14 days post partum contained a component of lower electrophoretic mobility than had been previously noted. The sedimentation patterns of the pig wheys reveal two definite heterogeneous areas for the proteins of lower sedimentation constant.

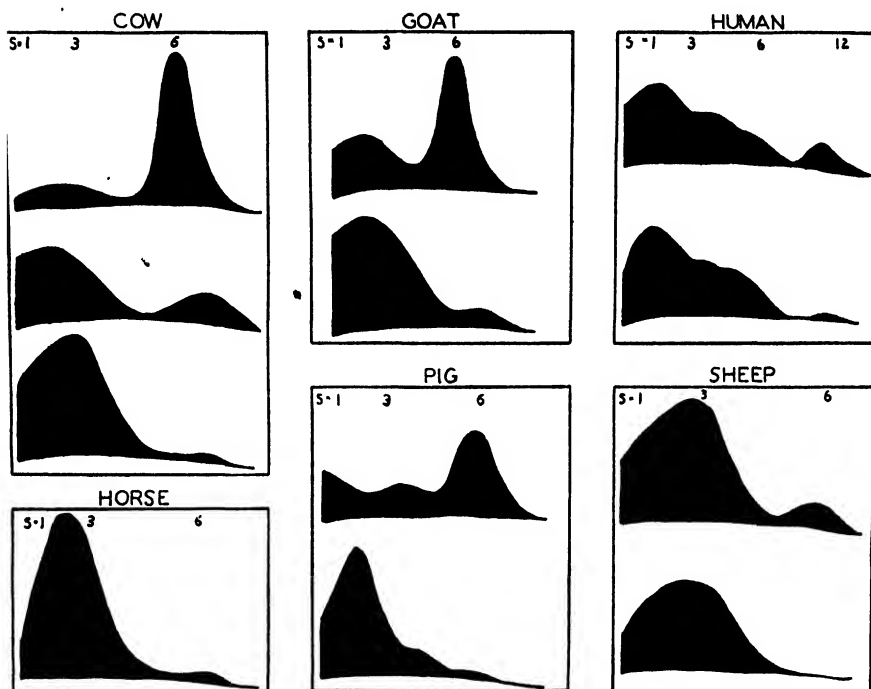


FIG. 8. Sedimentation diagrams of milk wheys at various times post partum. The earliest samples shown at the top of series and times post partum for various animals are as follows: cow, 0 and 2 days, aged; horse, 11 days; goat, 0 and 2 days; pig (Poland-China), 0 and 9 days; human, 1, 2, and 90 days; sheep, 7 and 35 days. Sedimentation constants in Svedberg units form the abscissae.

Two samples of sheep milk were studied. The sample taken 7 days post partum gave a whey which showed larger amounts of the globulin constituent compared to the later sample. The electrophoretic patterns of the whey proteins of this species, Fig. 6, are quite similar to those of the goat wheys. The sedimentation patterns of the sheep wheys shown in Fig. 8 reveal the presence of slightly larger amounts of protein of higher sedimentation constant in the sample taken at the earlier postpartum time.

The wheys from two samples of horse milk taken relatively late post

partum showed almost identical electrophoretic composition, as is seen by the results in Table II. However, the electrophoretic pattern shown in Fig. 7 is distinctly different from that of the other animals. There is an electrophoretic component of very low mobility present in these wheys. A component in the whey of a Chester white sow (Fig. 5), taken 14 days post partum, has a similar mobility. The sedimentation diagram for horse whey (Fig. 8) reveals less heterogeneity in the proteins of low sedimentation constant than is characteristic of the other animal wheys.

DISCUSSION

The changes in the nitrogen content found in samples of milk of various animals at different times post partum correspond in general with values reported in the literature. A portion of the nitrogen of milk wheys is known to be dialyzable and to be made up of nitrogenous constituents other than protein. Thus, in the human milk sample collected 1 to 2 days post partum, 31.3 per cent of the whey nitrogen was found to be dialyzable. This is in agreement with the high non-protein nitrogen value of 15 to 25 per cent reported for normal human whole milk by various investigators (8-11). Cow's milk has been reported to have much lower non-protein nitrogen values than human milk (9).

Colostrum wheys are characterized by the presence of large amounts of proteins of low electrophoretic mobility. In these wheys there is also found a marked increase in the amount of the protein component of sedimentation constant $s_{20w} = 6$ Svedberg units. Smith (12) has found that the immune globulins of bovine colostrum which he has separated by fractionation and which correspond to our Component 1 consist largely of molecular kinetic units having a sedimentation constant of $s_{20w} = 7$ Svedberg units. The corresponding immune globulins of our unfractionated wheys have been found to give somewhat lower sedimentation constants. Pedersen (13) has reported a normal lactoglobulin of milk to have a sedimentation constant of $s_{20w} = 7.0$ to 7.4 Svedberg units, although a fraction made up largely of this protein was found to give a somewhat lower value. The greater part of normal bovine whey proteins consists of the lactoglobulin of Palmer and the albumin of Kekwick, which have sedimentation constants (s_{20w}) equal to approximately 3 and 2 Svedberg units respectively. A component not shown in Fig. 8 and possessing a sedimentation of $s_{20w} = 20$ Svedberg units makes up approximately 10 per cent of the proteins of bovine colostrum whey. So heavy a protein molecule has not been found in the serum γ -globulins of normal (5) or hyperimmunized (12) cows. However, Smith (12) has recognized such a component in the euglobulin fraction of the immune lactoglobulins of bovine milk and colostrum.

The electrophoretic patterns of the whey proteins are characteristic

of the species, as is true of the blood serum and plasma proteins (1, 2). Although some of the mobilities of the electrophoretic constituents of a given whey agree with those of certain plasma constituents of the same animal, as determined previously (1), there seems to be little relation between the plasma and whey proteins from an electrophoretic standpoint. The large amounts of protein in the wheys from the first postpartum milk samples, which we have designated Component 1 do, however, possess an electrophoretic mobility much like that of the immune globulins of the blood plasma.

The young of various species receive antibodies from different sources. The placental structure of humans is essentially one cell thick and allows the antibody protein to pass from the maternal blood to the young *in utero*. However, it appears that young foals, kids, lambs, pigs, and calves acquire these proteins from the colostrum (14-17). In these animals the placental membranes contain three or more cell layers (18). This difference may be reflected in the failure of human whey to show the high level of protein Component 1 which is observed in the early postpartum wheys of the pig, goat, and cow.

SUMMARY

The whey proteins of various animals show marked differences in electrophoretic composition and, as in the case of the plasma proteins, are characteristic for a given species. Marked changes in the protein content of the milk at various times post partum are indicated by changes in the electrophoretic and sedimentation patterns.

The author wishes to acknowledge the technical assistance of Mr. E. H. Hanson, Mrs. Alice McGilvery, and Miss M. S. Morris. The helpful suggestions of Dr. J. W. Williams are greatly appreciated. Various samples of milk were received through the courtesy of Dr. R. H. Grummer and Dr. R. C. Herrin. The work was supported in part by grants from the Wisconsin Alumni Research Foundation and the United States Public Health Service.

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LETTERS TO THE EDITORS

THE PRESENCE OF HIGH β -GLUCURONIDASE ACTIVITY IN CANCER TISSUE*

Sirs:

β -Glucuronidase is believed to function in the "metabolic conjugation" of the estrogenic hormones.¹ In view of the large amount of experimental

Distribution of β -Glucuronidase in Adenocarcinoma of Breast with Metastases to Axillary Lymph Nodes

Organ	Tissue	No of specimens studied,	Glucuronidase activity*
			<i>units</i>
Breast	Edge of lesion	1	450
"	Center of lesion	1	890
"	Uninvolved breast	2	168, 112
"	" skin over carcinoma	1	89
"	" fat	1	57
"	" muscle	1	98
Right axilla	Involved lymph nodes	2	1960, 3520
" "	Uninvolved lymph nodes	2	816, 572
Left "	Involved lymph node	1	3700
" "	Uninvolved lymph nodes	5	633, 510, 640, 365, 645

On March 28, 1947, a radical resection of the breast and right axilla was performed on Mrs. C. The lymph nodes of the left axilla were removed on April 4. The presence or absence of carcinoma was confirmed histologically.

* A unit of β -glucuronidase is defined as 1 γ of phenolphthalein liberated per hour per gm. of wet tissue from 0.01 N phenolphthalein mono- β -glucuronide buffered at pH 4.5, 0.1 M acetate buffer, at 37.5°.

and clinical evidence which indicates a rôle of the estrogenic hormones in the production of malignant growth, the glucuronidase activity of cancerous tissues was studied. The data in this report are regarded as evidence of the involvement of β -glucuronidase in processes of malignant growth.

* Aided by a grant from the Otho S. A. Sprague Memorial Institute.

¹ Fishman, W. H., *J. Biol. Chem.*, **169**, 7 (1947).

Fresh tissue specimens removed at operation were divided; one-half was fixed in formalin for histological study and the other half was weighed, homogenized in water, and assayed for β -glucuronidase activity. Tissues excised from malignant neoplasms of various organs including breast, uterus, stomach, mesentery, abdominal wall, and esophagus were found to contain from 100 to 3600 per cent more glucuronidase activity than the uninvolved adjacent tissue. Metastases to lymph nodes from cancers originating in various organs including stomach, lung, breast, finger, colon, and esophagus likewise contained β -glucuronidase in higher concentration than the uninvolved lymph nodes. As an illustration, the table lists the enzymic activities of cancerous and non-cancerous portions of the breast and axillary lymph nodes removed at operation.

In view of the evidence^{1, 2} of a participation of β -glucuronidase in the metabolism of estrogens, the elevated glucuronidase in the tumor tissue may be interpreted as a metabolic response to the presence of high concentrations of estrogen or some closely related substance. Investigations now in progress are designed to test this hypothesis.

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A. J. ANLYAN

Received for publication, May 2, 1947

¹ Fishman, W. H., and Fishman, L. W., *J. Biol. Chem.*, **152**, 487 (1944).

THE RÔLE OF BIOTIN IN BICARBONATE UTILIZATION BY BACTERIA

Sirs:

In the present study of the mechanism by which biotin affects the synthesis of aspartate,¹ oxalacetate has been found capable of promoting growth of *L. arabinosus* 17-5 on media² deficient in both biotin and aspartic acid (Table I). It therefore seems likely that the biotin-deficient organism

TABLE I

Volume of media	Addition to basal medium	Ml. of acid produced in 36 hrs *	
		Low biotin† media	High biotin† media
Macro, 10 ml.	None	1.3	10.0
	Oxalacetate, m/135‡	4.5	10.0
	DL-Aspartate, m/670	8.5	10.0
Semimicro, 2 ml.	None	0.66	4.9
	Oxalacetate, m/135‡	2.7	4.8
	DL-Aspartate, m/670	5.0	4.9

* Macro-titrated with 0.1 N NaOH; semimicro-titrated with 0.04 N NaOH.

† Media containing 0.05 and 2.0 micromicrograms of biotin per ml respectively.

‡ Sterile solution added aseptically at time of inoculation.

can convert oxalacetate to aspartate by transamination. The extreme lability of oxalacetate may account for the fact that it is not as effective as aspartate in promoting growth on low biotin media. Pyruvate, succinate, fumarate, L-malate, and α -ketoglutarate did not support growth.

The synthesis of oxalacetate by a number of heterotrophic organisms involves the condensation of pyruvate and CO₂,³ and Lyman *et al.*⁴ have recently shown the necessity of CO₂ for the synthesis of aspartic acid by lactobacilli. Since the lactic acid bacteria are known to produce pyruvate during the fermentation of glucose, it seemed possible that the inability of biotin-deficient organisms to synthesize aspartate lay in their failure to

¹ Koser, S., Wright, M., and Dorfman, A., *Proc. Soc. Exp. Biol. and Med.*, **51**, 204 (1942). Stokes, J. L., Larsen, A., and Gunness, M., *J. Biol. Chem.*, **167**, 613 (1947).

² Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).

³ Werkman, C., and Wood, H. G., *Bot. Rev.*, **8**, 1 (1942).

⁴ Lyman, C. M., Mosley, O., Wood, S., Butler, B., and Hale, F., *J. Biol. Chem.*, **167**, 177 (1947).

condense pyruvate and CO_2 to produce oxalacetate. The data in Table II show that bicarbonate elicits no appreciable growth response in a biotin-low medium devoid of aspartic acid, and that bicarbonate greatly stimulates growth in the presence of biotin.

TABLE II

Additions to basal medium	Galvanometer readings*			
	Low biotin medium†		High biotin medium†	
	12 hrs.	18 hrs.	12 hrs.	18 hrs.
None	89	87	80	52
Bicarbonate, m/270‡ . . .	89	86	66	43
Oxalacetate, m/270‡ . .	86	81	59	34
Aspartate, m/670 . . .	82	72	66	31

* Turbidity was measured with an Evelyn colorimeter and Filter 660; sterile media set at 100. The values are the average of triplicate tubes.

† Media containing 0.05 and 2.0 micromicrograms of biotin per ml. respectively.

‡ Sterile solution added aseptically at time of inoculation.

It is of interest that, on a high biotin medium, oxalacetate consistently stimulates early growth of *L. arabinosus* above the level obtained with aspartate or aspartate plus bicarbonate.

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Received for publication, April 16, 1947

INVOLVEMENT OF BIOTIN IN THE BIOSYNTHESIS OF OXALACETIC AND α -KETOGLUTARIC ACIDS

Sirs:

It has recently been shown that 2-oxo-4-imidazolidinecaproic acid¹ prevents the biosynthesis of biotin in *Escherichia coli* by competing with desthiobiotin for the enzyme system which converts desthiobiotin to biotin.² In the present investigation, it has been found that the antibacterial index for this competitive inhibition was increased 3-fold (from about 100 to 300) by exogenous α -ketoglutaric acid (300 to 1000 γ per 5 cc.).³ L-Glutamic acid produced a similar effect, but aspartic, succinic, fumaric, malic, and *cis*-aconitic acids were inactive. From our first discussion of *inhibition analysis*,⁵ it is apparent that substances having a sparing action on the product of the inhibited enzyme system such as to decrease the quantity of product necessary for a given amount of growth will cause a change in the antibacterial index. That α -ketoglutaric or glutamic acid exerts such a sparing effect is shown on addition of either of the metabolites to the growth medium by the decrease in the quantity of biotin from 0.03 to 0.003–0.01 γ per 5 cc. as the amount necessary to prevent the toxic action of 1 mg. of the inhibitor per 5 cc. of medium. From these data, it appears that the first system affected by decreased biotin synthesis in *E. coli* is the biosynthesis of α -ketoglutaric acid.

Since α -ketoglutaric acid is apparently involved in the biosynthesis of aspartic acid in *E. coli*,⁵ the desthiobiotin analogue and inhibitory aspartic acid analogues,^{4, 5} should be synergistic in preventing growth of *E. coli*. Such synergism with the desthiobiotin analogue was obtained both with hydroxyaspartic acid in the presence of pantothenic acid and with cysteic acid. This type of phenomenon suggests two blocked enzyme processes in sequence.

Results previously obtained by Garrison and Eakin⁶ in this laboratory

¹ The 2-oxo-4-imidazolidinecaproic acid was supplied by Dr. R. Duschinsky; the γ -(3,4-ureylenecyclohexyl)butyric acid by Dr. R. O. Roblin, Jr.

² Rogers, L. L., and Shive, W., *J. Biol. Chem.*, **169**, 57 (1947).

³ The testing methods have been previously described (see foot-notes 2, 4, 5). For *L. arabinosus*, aspartic acid was omitted from the basal medium, and biotin was supplied in concentration beginning at 0.01 γ per 5 cc. α -Ketoglutaric and oxalacetic acids were filtered and added aseptically; the latter was added in 5 equal parts at intervals during the incubation period. *E. coli* was incubated 17 hours at 37°; *L. arabinosus* 24 hours at 30°.

⁴ Shive, W., and Macow, J., *J. Biol. Chem.*, **162**, 451 (1946).

⁵ Ravel, J. M., and Shive, W., *J. Biol. Chem.*, **166**, 407 (1946).

⁶ Garrison, L. S., and Eakin, R. E., unpublished data.

indicate that biotin functions in yeast in the carboxylation of pyruvic acid to form oxalacetic acid. By analogy one might suppose that biotin also functions in the conversion of oxalsuccinic acid to α -ketoglutaric acid.

Further establishing this relationship of biotin to the synthesis of oxalacetic acid, an *inhibition analysis* of the competitive inhibition of growth of *Lactobacillus arabinosus*,³ obtained with γ -(3,4-ureylenecyclohexyl) butyric acid¹ displacing biotin, indicated that the antibacterial index was approximately 30,000 in the absence of aspartic acid or oxalacetic acid but increased to 300,000 in the presence of either metabolite (1 mg. per 5 cc.). Succinic, malic, or fumaric acid did not affect the toxicity of the inhibitor. In view of the sparing action of aspartic acid on biotin reported for this and other organisms,^{7, 8} it would appear that the change in antibacterial index on addition of oxalacetic acid or aspartic acid is due to a sparing action on the biotin coenzyme, the formation of which is prevented by the biotin analogue.

These results tend to bear out the conclusion of Garrison and Eakin⁶ that biotin functions in the carboxylation of pyruvic acid to oxalacetic acid.

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⁷ Koser, S. A., Wright, M. H., and Dorfman, A., *Proc. Soc. Exp. Biol. and Med.*, **51**, 204 (1942).

⁸ Stokes, J. L., Larsen, A., and Gunness, M., *J. Biol. Chem.*, **167**, 613 (1947).

UNIDENTIFIED GROWTH FACTORS FOR LACTOBACILLUS LACTIS IN REFINED LIVER EXTRACTS*

Sirs:

In an attempt to find a microorganism that might require a rat growth factor found in liver extracts and certain caseins¹ and in some foodstuffs,² it was noted³ that *Lactobacillus lactis* Dorner,^{4,5} required the presence of

Assay of LLD Factor in Liver Extracts in Presence of TJ Factor

Liver preparation	Amount of liver extract giving half maximum growth*
	mg.
Liver Fraction L (Wilson)	0.6
	ml.
1/60 unit oral liver extract, Company A	0.0119
1 " " " " " B	0.0093
3.3 units " " " " A	0.00435
5 " refined liver extract, Company B	0.0025
10 " " " " " "	0.00034
10 " " " " " A	0.00043
15 " " " " " B	0.00035
15 " " " " " A	0.00022

* Defined as equal to a galvanometer reading of 65 on the Evelyn colorimeter, Filter 620, when *Lactobacillus lactis* Dorner is grown for 66 hours at 37°, in a total volume of 10 ml. of amino acid basal medium containing 0.5 ml. of clarified tomato juice per tube.

two unidentified factors for growth in an amino acid basal medium containing all the synthetic B vitamins. One factor, present in clarified canned tomato juice (TJ), was also found in low amounts in casein and in many other substances, while the second heat-stable factor (LLD) was found in

* Scientific paper No. A 164. Contribution No. 2059 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry).

¹ Cary, C. A., Hartman, A. M., Dryden, L. P., and Likely, G. D., *Federation Proc.*, **5**, 128 (1946).

² Hartman, A. M., *Federation Proc.*, **5**, 137 (1946).

³ Shorb, M. S., *J. Bact.*; abstract in press.

⁴ Peterson, W. H., and Peterson, M. S., *Bact. Rev.*, **9**, 49 (1945).

⁵ The author is indebted to Dr. Morrison Rogosa, Bureau of Dairy Industry, United States Department of Agriculture, Washington, D. C., for this culture. *Lactobacillus lactis* Dorner is No. 8000 of the American Type Culture Collection.

highest concentrations in the liver extracts active for rat growth, but not in casein or casein hydrolysates.³

Assays for the LLD factor in crude and refined liver extracts of the type used for intramuscular injection in the treatment of pernicious anemia show that the LLD factor is apparently concentrated in the refined extracts in almost linear relationship to the potency of the extracts for effecting remission of symptoms in pernicious anemia. This relationship, as shown in the table, suggests that the LLD factor might be the active principle in pernicious anemia. It is known that synthetic folic acid is not the active principle in pernicious anemia concentrates.⁶

TJ factor is present in variable amounts in liver extracts, but is generally more concentrated in the crude liver extracts than in the refined extracts. While the TJ factor may not have any function in the treatment of anemias, it may be that both TJ and LLD factors are of fundamental importance, since both factors are required together for the growth of *L. lactis* Dorner.

The relationship of the LLD and TJ factors to those for other bacteria and animals is not clear at present. A third factor appears to be synthesized by *L. lactis* Dorner and is found in certain fractions obtained from Wilson's Liver Fraction L. Inhibitory substances are present in many natural materials, in some tomato juice preparations, and liver fractions. Because of many variables influencing the growth of *L. lactis* Dorner, further work is needed in the separation of the various factors before accurate assay methods can be worked out. *L. lactis* Dorner should be of value in the further study of these factors and their relation to anemias.

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* Frommeyer, W. B., Jr., and Spies, T. D., *Am. J. Med. Sc.*, **213**, 135 (1947).

THE ANTISTREPTOMYCIN ACTIVITY OF LIPOSITOL*

Sirs:

In an earlier report¹ it was shown that the antibacterial action of streptomycin on *Eberthella typhosa* and *Staphylococcus aureus* was greatly decreased when the bacteria were grown in Difco brain-heart infusion. In a continuation of this work it was found that a methanol extract of the brain-heart infusion contained the streptomycin-inhibiting factor and that Difco heart infusion had no activity. In order to study the nature of the factor, Difco Casamino acids medium was used as the base medium. It was first determined that the vitamin content of Casamino acids medium and even added vitamin did not carry the factor. Comparison of peptone, neo-peptone, proteose peptone, peptone 3, tryptose, tryptone, trypticase, phytone, and Liver Fraction L indicated that phytone (a plant peptone) was the most active. In order to save time a shorter assay method was devised at this point. The modified procedure consisted of adding 2.5 ml. of double strength base medium to a solution of the material to be assayed in 2 ml. of water. The final solution was sterilized and cooled. Streptomycin (0.5 ml.) of the desired concentration and 1 standard drop of a 24 hour broth culture of the test organism were then added. The total volume was 5 ml. in all tests and the amount of streptomycin added in the tests with *E. typhosa* was 200 units per ml., or a total of 1000 units, and with *S. aureus* 50 units per ml., or a total of 250 units. Incubation was at 37° for 15 hours. The presence or absence of turbidity was used as the measure for the presence of the active factor. Final checks were made by plating 1 ml. of the solution after the 15 hour incubation interval. Several different concentrations of the assay material were tested in order to determine the minimum amount that gave effective inhibition of streptomycin.

The original brain-heart infusion was active at a level of 25 to 50 mg. A special Difco brain infusion² gave inhibition at a much lower level. At this point a systematic fractionation of the active substance from brain was undertaken. As was expected, aqueous extracts were ac-

* The authors wish to express their appreciation to the Abbott Laboratories, Eli Lilly and Company, Parke, Davis and Company, and The Upjohn Company for a grant in support of this work.

¹ Wallace, G. I., Rhymer, I., Gibson, O., and Shattuck, M., *Proc. Soc. Exp. Biol. Med.*, **60**, 127 (1945).

² Difco brain infusion, experimental, S4472. The authors wish to express their appreciation to Dr. H. W. Schoenlein and the Difco Laboratories, Inc., for this material.

tive. To our surprise, however, ether removed the major portion of the activity from an acetone-dried brain preparation. The ether-soluble material on extraction with water yielded a fraction active at a level of 1 mg. (*S. aureus*). These data suggested that the active substance might be lipositol.² The solubility of the substance in both ether and water and its occurrence in brain and in phytone (partially derived from soy bean) pointed in this direction. Furthermore the presence in lipositol of an inositol-galactose structure suggested the possibility of a metabolite-antimetabolite relationship with streptomycin. Dr. Woolley very kindly provided us with a sample of soy bean lipositol. On testing, this substance proved to be active at the extremely low level of 0.001 mg. (*S. aureus*). In other words the antibacterial activity of 50 units (0.06 mg.) of streptomycin per ml. was suppressed by 0.0002 mg. of lipositol per ml.

The data raise some very interesting questions regarding the rôle of lipositol in nutrition and its relationship to the antibacterial action of streptomycin. These problems are being investigated in our laboratories at the present time, and the effect of other phospholipides on streptomycin is being studied.

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² Woolley, D. W., *J. Biol. Chem.*, **147**, 581 (1943).

DISTRIBUTION OF LABELED CARBON IN SUGAR FROM BARLEY*

Sirs:

We have recently prepared radioactive sugars from barley seedlings^s which assimilated $C^{14}O_2$.¹ It is of importance to know the distribution of the labeled carbon in the sugars formed under these conditions.

Two microbiological methods were used in the degradation: (1) the fermentation of the sugars by yeast and (2) by *Lactobacillus casei*. The scheme employed with the latter organism is essentially that used by Wood *et al.*²

The primary modification in the scheme is the oxidation of the lactic acid to carbon dioxide and acetic acid, rather than acetaldehyde, followed

Source	Sugar	Organism	Carbons 1,6	Carbons 2,5	Carbons 3,4
With roots	Hydrolyzed sugar	Yeast		1.00	1.96
	Crystalline glucose	<i>L. casei</i>	0.83	1.00	1.60
Without roots	Non-hydrolyzed	" "	0.41	1.00	2.95
	Crystalline glucose	" "	0.68	1.00	2.61

by the conversion of the barium acetate to acetone and thence to iodoform. All final counts were made as $BaCO_3$.

In the yeast fermentation method, the procedure was identical to that depicted above, following the oxidation of the alcohol to acetic acid.

Sugars from plants with and without roots were investigated, and the results appear to indicate definite differences in isotope distribution. In two experiments the glucose was prepared from sugar extract by crystallization. In the two other experiments, one sample of sugar was acid-hydrolyzed, while the other was used without further treatment.

The relative activities of the carbons, based on position 2,5 arbitrarily assigned equal to 1.00, are tabulated. It will be noted that with the methods used it is impossible to distinguish between carbons the analysis of which occurs in pairs because of the tautomerism of the trioses formed in the primary cleavage of the hexose. It is possible that they are not equal, and experiments are in progress to determine this point.

* This paper is based on work performed under contract No. W-7405-Eng-48 with the Atomic Energy Commission and the University of California.

¹ Aronoff, S., Benson, A. A., Hassid, W. Z., and Calvin, M., *Science*, in press.

² Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159**, 475 (1946).

Assuming that hexoses are formed from triose phosphates, these results indicate that the carbon distribution in the trioses is not uniform, and that the activity is greatest in the terminal carbon atoms which eventually become carbons 3 and 4 in the hexose.

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ORNITHINE-CATALYZED UREA FORMATION IN LIVER HOMOGENATE*

Sirs:

Heretofore attempts to obtain synthesis of urea from ammonia (and carbon dioxide) in cell-free extracts have been unsuccessful. We have found the reaction to proceed in guinea pig liver homogenate. The following is the reaction mixture which has given the highest yields obtained so far: L-ornithine (0.00075 M), ammonia (0.0025 M), L-glutamate (0.01 M), oxalacetate (0.005 M), ATP (0.00025 M), and 0.33 gm. of homogenized liver in a final volume of 3.5 ml. The following are typical increases in urea over the blank observed in 1 hour, in micrograms: ornithine + ammonia 0, glutamate + oxalacetate 0, glutamate + oxalacetate + ornithine 49, glutamate + oxalacetate + ammonia 105, glutamate + oxalacetate + ornithine + ammonia 317, glutamate + oxalacetate + ornithine + ammonia (without ATP) 150.

The buffer solution consisted of 90 parts of the phosphate-saline solution of Cohen and Hayano¹ and 10 parts of 0.13 per cent sodium bicarbonate. The liver was homogenized by the method of Potter and Elvehjem.² The reaction was carried out at 38° and pH 7.5 under 5 per cent carbon dioxide and either 95 per cent oxygen or nitrogen.

Urea was determined by two methods: the xanthidrol method of Engel and Engel³ or the colorimetric diacetyl monoxime method of Barker⁴ before and after digestion with urease. The two methods gave similar results.

Addition of pyruvate (0.01 to 0.005 M) to the above reaction mixture did not give higher yields of urea. The yield was smaller when oxalacetic acid was omitted, and greater when fumarate (0.05 M) was substituted for it. Glutamine was as effective, but not more so than equivalent concentrations of glutamate and ammonia.

Urea formation in any of the above reaction mixtures was completely inhibited by anaerobiosis and by 0.0036 M sodium arsenate.

Analyses of the absorption curves of the color obtained with diacetyl monoxime after digestion with urease indicated that citrulline and other chromogenic material were formed during the reaction. By omitting ATP from the reaction mixture less urea was formed and the total residual

* This work is a part of that done under contract with the Office of Naval Research, United States Navy Department.

¹ Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **166**, 251 (1946).

² Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).

³ Engel, M. G., and Engel, F. L., *J. Biol. Chem.*, **167**, 535 (1947).

⁴ Barker, S. B., *J. Biol. Chem.*, **152**, 453 (1944).

chromogenic material after urease digestion (designated as "citrulline" for the time being) was doubled. Expressed in terms of citrulline standards, ornithine plus ammonia gave at 38° per 0.33 gm. of liver per hour, an increase in "citrulline" of 80 γ ; ornithine plus ammonia plus glutamate 560 γ ; glutamate with either ornithine or ammonia omitted 200 γ or less. Pyruvate was as effective and oxalacetate less effective than glutamate.

Anaerobiosis or 0.0036 M sodium arsenate inhibited the formation of "citrulline." Anaerobiosis also inhibited the conversion of citrulline to urea (the reaction mixture contained added glutamate and ATP). On the other hand 0.0036 M sodium arsenate did not inhibit urea formation from citrulline. The conversion of citrulline to arginine in rat kidney slices is completely inhibited by 0.0036 M arsenate.⁵ It is difficult to reconcile the two observations with the view that conversion of citrulline to arginine is the obligatory next step in the path to formation of urea. It would appear that in guinea pig liver homogenate citrulline may give urea by another path as well as via its immediate conversion to arginine.

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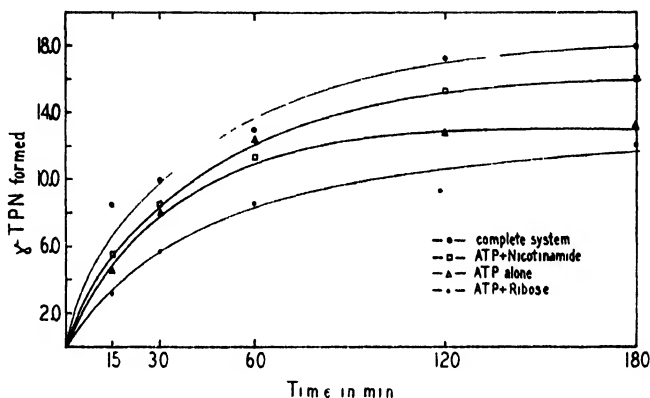
⁵ Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **141**, 717 (1941).

ENZYMIC SYNTHESIS OF TRIPHOSPHOPYRIDINE NUCLEOTIDE IN CELL-FREE EXTRACTS*

Sirs:

Knowledge of the mechanism of synthesis of diphosphopyridine nucleotide and triphosphopyridine nucleotide is lacking, although the enzymic inter-conversion of the two substances has been demonstrated.¹

We have found that aqueous extracts of pigeon liver acetone powder, dialyzed for 3 days against 0.025 M phosphate buffer, pH 7.4, contain a thermolabile enzyme system capable of the synthesis of TPN when ATP



The reaction mixtures were incubated at 30° for a period indicated by the abscissa. After incubation the solution was placed in a boiling water bath for 1 minute, cooled, neutralized with 2 N KOH, and made up to 2 ml. final volume. The final concentrations of each of the components of this system were 0.13 M acetate buffer, pH 5.0 1.3×10^{-3} M nicotinamide, 1.3×10^{-3} M D-ribose, 2×10^{-3} M ATP, and 0.3 ml. of enzyme solution.

is added. Nicotinamide increases the quantity of TPN synthesized and a maximum effect is observed with a system containing added nicotinamide, ATP, and ribose. The original enzyme solution does not contain TPN or DPN. Microbiological assay for niacin before and after incubation of the enzyme solution with ATP indicates that all of the niacin originally present can be accounted for by the TPN synthesized.

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¹ von Euler, H., Adler, E., and Eriksen, S., *Z. physiol. Chem.*, **248**, 227 (1937). von Euler, H., and Adler, E., *Z. physiol. Chem.*, **252**, 41 (1938). Vestin, R., *Ark Kemi, Mineral. o. Geol.*, **12B** (1938).

When the enzyme solution is dialyzed against veronal buffer, pH 7.5, the effect of ATP and the synthesis of TPN can be demonstrated only if inorganic phosphate (0.025 M) is added. Adenylic acid can be substituted for ATP but increased amounts of inorganic phosphate are then required.

The synthesis of TPN in phosphate buffer is inhibited by 10^{-4} M NaCN and 10^{-3} M NH_2OH (to about 50 per cent) but is not affected by 10^{-3} M iodoacetate or 2×10^{-2} M NaF.

TPN was determined by use of the system described by Haas,² quantities of TPN from 0.0 to 5.0 γ of TPN being determined with an accuracy of ± 0.1 γ . The test substances were prepared as previously described.³

Data from a typical experiment are presented in the figure.

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² Haas, E., *J. Biol. Chem.*, **155**, 333 (1944).

³ Altman, K. I., *J. Biol. Chem.*, **166**, 149 (1946).

SYNERGISM BETWEEN INDOLEACETIC AND NICOTINIC ACIDS IN A PLANT GROWTH INHIBITION

Sirs:

Recent investigations¹ have demonstrated a metabolic relation between tryptophan and nicotinic acid in various animals. It has also been claimed that indoleacetic acid, a known plant metabolite of tryptophan,² may be pellagragenic in the rat.³ Because of the physiological importance of indoleacetic acid as a plant growth hormone, it was deemed advisable to investigate the possibility of a nicotinic acid-indoleacetic acid interrelationship in plants.

TABLE I
Growth of Excised Asparagus Stem Tips in Dark, with and without IAA

Duration of experiment, days	No of tubes per treatment	Average total growth, mm per stem tip		
		Control	Plus 1 γ per cc IAA	Plus 10 γ per cc IAA
61	15	61.6 \pm 7.0*	45.6 \pm 7.4	7.6 \pm 1.5
29	10	34.9 \pm 3.9	17.0 \pm 4.7	6.0 \pm 0.86
41	10	30.0 \pm 5.7	8.3 \pm 3.1	1.7 \pm 0.14

* Standard error.

In these experiments, sterile cultures of excised asparagus stem tips were used as the experimental material. Loo⁴ has shown that such stem tips are capable of potentially unlimited growth on agar containing only minerals and sucrose. Asparagus seeds (variety Mary Washington) were surface-sterilized by HgCl₂ and germinated in sterile Petri dishes on 1 per cent agar. After 2 to 3 weeks, 8 to 10 mm. portions of the stem tip were excised and implanted in test-tubes containing 5 cc. of Loo's medium. The tubes were incubated in the dark at 22°, growth in length being measured every 3 days.

Whereas indoleacetic acid (IAA) tends to stimulate growth in the light, it acts as a potent growth inhibitor in the dark (Table I). Nicotinic acid

¹ Krehl, W. A., Sarma, P. S., Teply, L. J., and Elvehjem, C. A., *J. Nutr.*, **31**, 85 (1946). Rosen, F., Huff, J. W., and Perlzweig, W. A.; *J. Biol. Chem.*, **163**, 343 (1946). Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, **167**, 293 (1947). Schweigert, B. S., Pearson, P. B., and Wilkening, M. C., *Arch. Biochem.*, **12**, 139 (1947). Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J., *Federation Proc.*, **5**, 154 (1946).

² Wildman, S. G., Ferri, M. G., and Bonner, J., *Arch. Biochem.*, **13**, 131 (1947).

³ Kodicek, E., Carpenter, K. J., and Harris, L. J., *Lancet*, **2**, 491 (1946).

⁴ Loo, S. W., *Am. J. Bot.*, **32**, 13 (1945); **33**, 156 (1946).

(NA), itself without any marked effect on growth rate in the light or dark, will greatly enhance the inhibitory effect of IAA in the dark when the latter is supplied at a concentration of 1 γ per cc. (Table II). No additional growth inhibition is produced by NA when IAA is supplied at 10 γ per cc.

TABLE II
Effect of Nicotinic Acid upon Indoleacetic Acid-Induced Growth Inhibition

Duration of experiment, days	No. of tubes per treatment	Average total growth, mm. per stem tip			
		Control	Plus NA(0.5 γ per cc.)	Plus IAA(1 γ per cc.)	Plus NA(0.5 γ per cc.) + IAA(1 γ per cc.)
70	10	44.5 \pm 4.3*	44.7 \pm 9.4	40.9 \pm 9.1	28.4 \pm 5.8
67	10	50.7 \pm 9.1	51.7 \pm 8.9	40.3 \pm 7.0	23.8 \pm 7.8
37	10	15.3 \pm 6.1	15.1 \pm 4.4	2.3 \pm 0.5	0.88 \pm 0.09

* Standard error

It therefore seems probable that NA is involved in the growth-regulatory effects produced in the plant by IAA, either by being metabolized to an IAA precursor or by participation in some intermediary reaction necessary for IAA activity.

Experiments are now in progress to determine whether NA may similarly enhance the stimulatory effect of IAA in light-grown asparagus stem tips.

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THE METABOLISM OF ACETYLAMINO ACIDS*

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(Received for publication, March 21, 1947)

The feeding to an animal of various amines, *e.g.* α -amino- γ -phenylbutyric acid, *p*-aminobenzoic acid, or sulfanilamide, results in the excretion of their N-acetyl derivatives. The rôle which the acetylation reaction plays in the amination of keto acids has been extensively discussed by Knoop (1) and by du Vigneaud *et al.* (2, 3). Much evidence concerning the sources of acetyl groups in biological acetylations has been secured during recent years. Two independent mechanisms of acetylation, one involving acetic acid and one involving pyruvic acid, have been demonstrated (4-6). These investigations have been facilitated by the inability of animal tissues to metabolize the acetyl derivatives of various amino compounds (here referred to as "foreign amines") alien to normal physiology. *p*-Aminobenzoic acid, in abnormally large doses, behaves as a foreign amine in that it is excreted in the form of its acetyl derivative.

Though the presence of acetyl derivatives of natural amino acids cannot well be demonstrated, as these compounds are rapidly metabolized, the finding, recently reported from this laboratory (7), that L-leucine and L-phenylalanine are acetylated by acetic acid in the presence of rat liver slices indicates that acetylation may constitute a normal process in the metabolism of natural amino acids.

The present investigation was designed to obtain more information on the metabolic behavior of the acetyl derivatives of natural amino acids. For this purpose, the deuterioacetyl derivatives of a number of amino acids were prepared and fed to rats. If these compounds underwent deacetylation *in vivo*, the liberation of acetic acid or acetyl should become evident by formation of the isotopic acetyl derivatives of foreign amines. Indeed, addition to the diet of rats of the labeled acetyl derivatives of glycine, L-alanine, L-glutamic acid, and L-leucine, in conjunction with either *p*-aminobenzoic acid or α -amino- γ -phenylbutyric acid, led to the excretion of labeled

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acetyl. However, the isotope levels in the excreted acetyl products differed markedly, depending on the type of foreign amine administered; much higher isotope concentrations were found in the acetyl group of the α -amino- γ -phenylbutyric acid than in that of the *p*-acetylaminobenzoic acid.

Under identical conditions, the deuterioacetyl derivatives of D-alanine, D-leucine, and of sarcosine failed to give rise to the excretion of isotopic acetyl groups. ϵ -N-Deuterioacetyllysine, though a source of labeled acetyl, showed a behavior different from that of α -N-acetyl amino acids.

EXPERIMENTAL

Preparation of Deuterioacetyl amino Acids—A mixture of 0.1 mole of anhydrous sodium deuterioacetate with 0.1 mole of *p*-toluenesulfonyl chloride was heated at 220° (8). The deuterioacetic anhydride which distilled was used directly for acetylation.

The amino acids were dissolved in 2 equivalents of NaOH and 1 equivalent each of deuterioacetic anhydride and of NaOH was gradually added to the ice-cooled solution with stirring. Stirring was continued for 20 minutes, when the solution was filtered and acidified with dilute H₂SO₄. The acetyl derivatives were recrystallized from water, except in the case of acetylalanine and acetylsarcosine with which the solvents used for recrystallization were ethyl acetate and acetone respectively. ϵ -N-Acetyl-L-lysine was prepared by the method of Neuberger and Sanger (9). The deuterioacetic anhydride contained 60.0 atom per cent deuterium in all cases except in that employed for the preparation of acetyllysine for which the value was 27.0 atom per cent. The analytical values of the acetyl compounds are given in Table I. The calculated nitrogen values for the compounds are corrected for the deuterium content of the acetyl group.

Feeding Experiments—Male white rats weighing about 200 gm. were kept on a normal stock diet to which was added the neutralized solution of the acetyl amino acid and either *p*-aminobenzoic acid or α -amino- γ -phenylbutyric acid. The quantities of test substances administered are indicated in Tables II to IV. The isolation from the urine of *p*-acetylaminobenzoic acid was carried out as described before (6).

In one of the experiments in which the animals received 0.8 mm per 100 gm. of acetylglycine, cholesterol isolated from carcasses was found to contain 0.13 atom per cent excess deuterium. This compares with a value 0.14 per cent after the administration of an equivalent amount of deuterioacetic acid for the same period of time. Acetylglycine is thus as effective a precursor for cholesterol as is acetate.

One of the preparations of acetylglycine contained 2.31 atom per cent N¹⁵; when this was fed with *l*- α -amino- γ -phenylbutyric acid, the isotope concentration in the excreted α -acetyl amino- γ -phenylbutyric acid was 0.010

atom per cent N^{15} . When the same experiment was repeated with *d*-amino-phenylbutyric acid instead of the *l* isomer, the excreted acetyl derivative contained 0.055 atom per cent excess N^{15} . These values are so low as to indicate little or no direct utilization of glycine nitrogen. During the conversion of the *d* to the *l* isomer, the intermediate keto acid is aminated, some of the nitrogen arising from the labeled glycine fed. For this reason the N^{15} concentration in the α -acetyl-amino- γ -phenylbutyric acid is higher after administration of the *d* isomer than after the *l* isomer.

TABLE I
Analytical Constants of Acetyl-amino Acids

Compound	N (Kjeldahl)		M p.	[α] _D
	Theory	Found		
	per cent	per cent		
Acetyl-glycine	11.6	11.6	208-209	
Acetyl-L-leucine	8.1	7.9	189-190	-22.5 (2% in absolute ethanol)
Acetyl-D-leucine	8.1	8.0	190	+24 (1% in absolute ethanol)
Acetyl-L-glutamic acid	7.4	7.2	201	+4.7 (2% in N NaOH)
Acetyl-L-alanine	10.5	10.3	130-132	-60.2 (1% in H ₂ O)
Acetyl-D-alanine	10.5	10.4	131-132	+63.4 (1% " ")
Acetylsarcosine	10.5	10.4	138	
ϵ -N-Acetyl-L-lysine	*	*	249-250	+4.0 (4% " ")
			(Decomposition)	

* N (Dumas), theory 14.9, found 14.8. NH_2 -N (Van Slyke) theory 7.4, found 7.4.

When acetyl-D leucine was tested as a source of acetyl in conjunction with α -amino- γ -phenylbutyric acid, the acetyl-amino acid fraction from the urine required five recrystallizations from water before *l*- α -acetyl-amino- γ -phenylbutyric acid with the correct melting point was obtained. Apparently acetyl-D-leucine had been excreted unchanged, but it could not be isolated in pure form.

Reaction of Diacetyldiketopiperazine with L-Leucine and Deuterioacetate - Diketopiperazine was prepared by the method of Fischer (10) and converted to the diacetyl derivative according to Franchimont and Friedmann (11).

To an ice-cooled solution of 0.69 gm. (5.2 mm) of L-leucine and of 0.100 gm. of sodium acetate (54.0 atom per cent deuterium) in 2.65 ml. of 2 N NaOH was added 0.50 gm. (2.6 mm) of diacetyldiketopiperazine. The diketopiperazine which soon precipitated was filtered off and the filtrate

made acid to Congo red. The acid solution on prolonged extraction with ether yielded acetyl-L-leucine, m.p. 191°; N (Kjeldahl), calculated 8.1, found 8.0. The product contained no significant excess of deuterium (0.010 per cent).

DISCUSSION

From detailed analysis of the acetylation reaction, carried out in this laboratory, it was concluded that either acetic acid or a functional derivative of acetic acid serves as the principal source of acetyl in the acetylation of both aromatic amines and foreign amino acids in the rat (6). The simultaneous feeding of deuterioacetate and either *p*-aminobenzoic acid or α -amino- γ -phenylbutyric acid leads to the excretion of acetyl derivatives whose isotope concentration is a function of the quantity of labeled acetic acid added to the diet. The data obtained conform with the equation

$$\frac{\text{Amount of acetate fed per 100 gm. rat weight}}{\text{Atom \% excess D in excreted acetyl group}} = K \quad (1)$$

This proportionality was taken to indicate the merging of labeled dietary acetate with relatively large quantities of endogenous acetic acid arising in intermediary metabolism, the resultant mixture being the immediate source of acetyl in the acetylation of foreign amines. If an acetylamino acid such as acetylglycine is rapidly hydrolyzed to yield acetic acid, its administration should result in the excretion of N-acetyl derivatives which have the same isotope concentration as those obtained after the feeding of an equivalent quantity of deuterioacetic acid. This is indeed the case when *p*-aminobenzoic acid is administered (Table II). Moreover, as in the case of deuterioacetate, variation of the dosage of labeled acetylglycine is followed by a proportional change in the isotope concentration of the acetyl group of acetyl-*p*-aminobenzoic acid, in accordance with equation (1) (Fig. 1). The similarity of the results obtained with acetic acid and with acetylglycine suggests that the process by which acetylglycine furnishes acetyl groups for *p*-aminobenzoic acid involves hydrolysis as an intermediate step and acetylation of the foreign amine by the mixture of isotopic and the non-isotopic endogenous acetic acid.

On the other hand, when α -amino- γ -phenylbutyric acid was administered with acetylglycine as the source of the acetyl group, the resultant acetylaminophenylbutyric acid had an isotope level of a different order of magnitude (Table II). Depending on the quantity of acetylglycine added to the diet, the excreted acetyl compound contained 5 to 20 times as much deuterium as is the case after the feeding of equivalent amounts of deuterioacetate. The type of curve obtained on plotting the quantity of deuterioacetylglycine fed per 100 gm. of rat weight against the isotope concentration

TABLE II

Atom Per Cent Deuterium in Acetyl Groups of Excreted Acetylmino Acids after Feeding of Deuterioacetylglucine and Deuterioacetate

Calculated for 100 atom per cent deuterium in acetate and in the acetyl group of acetylglucine. Aminophenylbutyric acid and *p*-aminobenzoic acid were fed in amounts corresponding to 0.4 mM per 100 gm. of rat weight per day.

Daily dose each of acetylglucine and acetate per 100 gm. rat weight	Deuterium concentration in acetyl group of acetylaminophenylbutyric acid after feeding		Deuterium concentration in acetyl group of acetyl- <i>p</i> -aminobenzoic acid after feeding	
	Acetate	Acetylglucine	Acetate	Acetylglucine
0.08	0.34	3.7, 4.0	1.5	3.3, 3.4
0.1				
0.2				
0.4	2.0, 2.1	12.7, 14.8	2.9, 2.0, 2.1	4.6, 5.3, 5.4
0.8	3.5	28.0	4.6	8.6, 8.0, 9.1
1.2	5.5			
1.6	8.5	29.0, 29.5	8.6, 9.7	

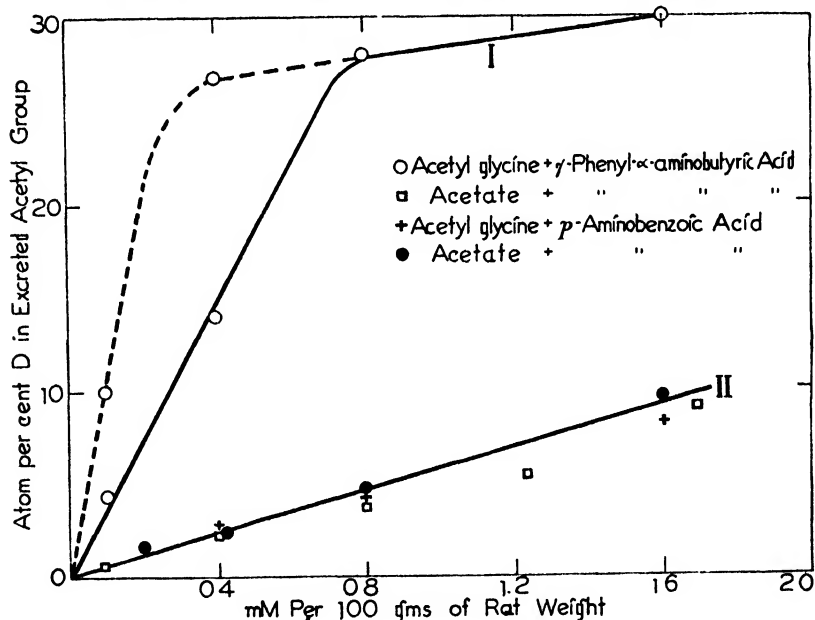


FIG. 1. Isotope concentrations in excreted acetyl group

of the acetyl group of the acetylaminophenylbutyric acid is markedly different from the linear relationship shown in the acetylation of the two types of foreign amines by deuterioacetate and also from the similar curve

obtained from the data on the acetylation of *p*-aminobenzoic acid by acetylglycine (Fig. 1). The isotope level in excreted acetyl does not increase proportionally with increasing dosage.

If hydrolysis to acetic acid had been the intervening step, acetylaminophenylbutyric acid should have contained an isotope concentration not exceeding that afforded by deuterioacetate, as in the acetylation of *p*-aminobenzoic acid. The much higher isotope level actually observed suggests that a different process of acetylation was responsible and that the acetyl group of acetylglycine underwent little dilution by endogenous acetic acid prior to its utilization in the acetylation of the α -amino- γ -phenylbutyric acid. If hydrolysis to acetic acid and glycine is excluded as an intermediary step, it becomes necessary to ascribe the observed process to a direct transfer of acetyl groups from one amino acid, glycine, to another, α -amino- γ -phenylbutyric acid, in the sense that during the process of acetylation acetyl arising from the donor acetyl amino acid did not pass through the stage of acetic acid or acetate ion.

There is other evidence to support the view that a direct transfer of acetyl groups is involved. In experiments in which labeled acetate was tested as a source of acetyl for either aromatic amines or foreign α -amino acids, the amount of foreign amine administered to the animal did not affect the isotope level of excreted acetyl (6). This was ascribed to the availability in the animal tissues of quantities of acetic acid which are always greatly in excess of those required to acetylate the foreign amine ingested by the animal. In the transfer reaction, the amount of foreign amine acetylated by this process should be limited by the amount of available acetyl donor. When the amount of foreign amine is large compared to that of acetylglycine, only a fraction of the former can receive its acetyl group by transfer and the isotopic level would be low. Conversely, reduction of the dosage of α -amino- γ -phenylbutyric acid should result in an elevation of the isotope level of excreted acetyl. The data in Table III and the dotted portion of Curve I in Fig. 1, which represents the effect of a reduced dosage of foreign amine at a constant level of acetylglycine, show this to be the case.

Acetylglycine, in contrast to acetic acid, affords markedly different isotope values in the acetyl groups of the two different types of foreign amines. The character of the amine appears to determine whether or not acetyl transfer takes place. We have previously found that the mechanism of acetylation which utilizes pyruvic acid as a source of acetyl is operative with the type of amine represented by α -amino- γ -phenylbutyric acid but not with sulfanilamide or *p*-aminobenzoic acid (6). The failure of pyruvic acid to provide acetyl groups for aromatic amines was ascribed to a process which required either a keto acid or an imino acid as reactant, compounds which cannot arise from amines in which the nitrogen atom is attached to an aromatic nucleus. On the other hand, good evidence exists that both the

corresponding imino and keto acids are formed biologically from α -amino- γ -phenylbutyric acid. It would not be inconsistent with the analytical data to assume that acetyl transfer forms an intermediate step in the process of acetylation which utilizes pyruvic acid. Pyruvic acid could condense with an imino acid to yield, after decarboxylation, an acetylamino compound which subsequently transferred the acetyl group to α -amino- γ -phenylbutyric acid.

In the reaction which effects the formation of an acetylamino acid by acetyl transfer, a simultaneous shift of amino acid nitrogen does not seem to take place. We have administered acetylglutamine which contained isotopic nitrogen in conjunction with both stereoisomers of α -amino- γ -phenylbutyric acid. The excreted acetyl derivatives contained so small an excess

TABLE III

Effect of Varying Dosage of α -Amino- γ -phenylbutyric Acid on Isotope Concentration of Excreted Acetyl Groups

Calculated for 100 atom per cent deuterium in the acetyl group of administered acetylglutamine.

mm administered per 100 gm. rat weight		Atom per cent deuterium in acetyl group of excreted acetylamino-phenylbutyric acid
α -Amino- γ -phenylbutyric acid	Deuterioacetylglutamine	
0.4	0.1	3.7, 4.0
0.1	0.1	9.3, 10.6
0.4	0.4	12.7, 14.8
0.1	0.4	26.0, 27.5

of N¹⁵ as to exclude a simultaneous transfer of nitrogen from acetylglutamine. In the case of *d*- α -amino- γ -phenylbutyric acid, which was converted to the acetyl derivative of the *l* acid, presumably by way of the keto acid, any utilization of glycine nitrogen for the reamination of the keto acid should have been readily detectable.

The findings, which are most readily explained as resulting from a transfer reaction, have been obtained largely with acetylglutamine as the test substance. The labeled acetyl derivatives of L-alanine, L-leucine, and L-glutamic acid approached acetylglutamine with respect to their ability to afford higher isotope concentrations in the acetyl group of α -acetylamino- γ -phenylbutyric acid than would result from equivalent quantities of deuterioacetate (Table IV). Two pathways of metabolism probably exist for the acetylamino acids, acetyl transfer and hydrolysis to acetic acid. The quantitative differences shown by the various acetylamino acids in the transfer reaction may be the result of differences in hydrolytic rates in competition with acetyl transfer.

Acetyl-D-leucine and acetyl-D-alanine failed to yield deuterioacetyl

to α -amino- γ -phenylbutyric acid. Some acetyl-D-leucine apparently was eliminated unchanged in the urine. The present findings are in accord with the early observations by Knoop (1) and du Vigneaud and Irish (2) that the acetyl derivative of D-phenylalanine is metabolically inert. This seems to be true also for the D isomer of the non-essential amino acid alanine.

As a representative amino acid derivative in which the acetyl group is not attached to the α -amino group, ϵ -N-acetyllysine was chosen for study. The compound yielded acetyl for the acetylation of α -amino- γ -phenylbutyric acid but only to the same extent as would have resulted from the feeding

TABLE IV

Atom Per Cent Deuterium in Acetyl Groups of Acetyl-p-aminobenzoic Acid and Acetyl-aminophenylbutyric Acid after Feeding of Labeled Acetylmino Acids

Calculated for 100 atom per cent deuterium in the acetyl group of the administered acetyl amino acid. All acetylmino acids were fed in quantities corresponding to 0.8 mm per 100 gm. of rat weight. The animals received 0.4 mm per 100 gm. of aminophenylbutyric acid.

Acetylmino acid	Acetyl group of acetylaminophenyl- butyric acid	Acetyl group of acetyl-p-aminobenzoic acid
Acetate	3.6	4.5
Acetyl-glycine	28.0	4.4
Acetyl-L-alanine	15.4	
Acetyl-D-alanine	0.97	
Acetyl-L-glutamic acid	17.3	
Acetyl-L-leucine	13.0	
Acetyl-D-leucine	0.2	
Acetylsarcosine	0.3	0.2
ϵ -N-Acetyl-L-lysine	4.9	

of an equivalent amount of deuterioacetate. Hydrolysis to lysine and acetic acid was probably the intermediate step. Neuburger and Sanger (9), who found ϵ -N-acetyllysine to be nutritionally equivalent to lysine for growing rats, concluded that free lysine was regenerated from this acetyl derivative. Interestingly, the α -N-acetyl derivative of the same amino acid is incapable of supporting the growth of rats on a lysine-deficient diet.

Acetylsarcosine was entirely ineffective as a source of acetyl for either type of foreign amine. Acetylsarcosine, in contrast to sarcosine which is known to be readily demethylated to glycine (12, 13), appears to be metabolically inert.

The data obtained in the present investigation indicate that the α -N-acetyl derivatives of those amino acids which are normal constituents of proteins provide acetyl groups for the acetylation of α -amino- γ -phenyl-

butyric acid by means of a process which does not involve the intermediary formation of acetic acid. In the course of the reaction, the isotope concentration of the acetylamino acid undergoes but little dilution, suggesting a direct transfer or shift of acetyl groups from one amino acid to another. Acetyl compounds derived from the amino acids of unnatural steric configuration are metabolically inert. Only α -amino acids, but not aromatic amines, can accept acetyl groups by transfer. The existence of a mechanism which specifically involves the acyl derivatives of naturally occurring amino acids lends further support to the view that acetylamino acids participate in the intermediary metabolism of amino acids.

Acetyl shift or acyl migration, either intermolecular or intramolecular, has been observed in a number of organic chemical reactions. *In vitro* model experiments which bear closely on the present biological observations are those described by Bergmann, du Vigneaud, and Zervas (14) on acetyl migration from diacetyldiketopiperazine to free amino acids. We have carried out an experiment analogous to that of Bergmann *et al.* by adding diacetyldiketopiperazine to an alkaline solution containing leucine and deuterioacetate. Diketopiperazine and acetylleucine are formed. As the acetylamino acid contained no excess of deuterium, acetylation must have taken place without participation of acetate ion. The compounds which *in vitro* act as donors in the transfer reaction have acetyl groups attached to nitrogen atoms linked to other acyl groups. It is possible that in the biological transfer of acetyl groups the acetylamino acid enters into linkages, such as peptide bonds, which would lead to similar activation.

SUMMARY

1. The deuterioacetyl derivatives of glycine, L- and D-alanine, L- and D-leucine, L-glutamic acid, sarcosine, and ϵ -N-acetyl-L-lysine have been prepared.

2. The simultaneous administration to rats of acetyl-L-amino acids containing a labeled acetyl group, together with α -amino- γ -phenylbutyric acid, led to the excretion in the urine of acetylaminophenylbutyric acid which contained a much higher isotope concentration than would have resulted from an equivalent quantity of deuterioacetic acid. This was not the case when *p*-aminobenzoic acid was the foreign amine. It is suggested that transfer of acetyl groups between two α -amino acids may take place.

3. The acetyl derivatives of the two unnatural amino acids tested are metabolically inert. ϵ -N-Acetyl-L-lysine yields acetyl but apparently not by direct transfer.

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THE FORMATION, RESOLUTION, AND OPTICAL PROPERTIES OF THE DIASTEREISOMERIC SULFOXIDES DERIVED FROM L-METHIONINE

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Toennies and Kolb (9) prepared γ -methylsulfinyl- α -aminobutyric acid (referred to hereafter as methionine sulfoxide) by oxidation of DL-methionine with hydrogen peroxide and called attention to the fact that the resolution of the four stereoisomers that comprised this product should be of interest because of possible metabolic significance. These authors also described the preparation of a crystalline picrate and later (unpublished work) found that the relatively low yields (about 58 per cent) were occasioned by a rather sharp separation into soluble and insoluble fractions, both of which yielded optically inactive material. If the first prefix denotes the configuration of the asymmetric carbon atom and the second prefix that of the sulfoxide group, the one fraction evidently consisted of an equimolar mixture of L-methionine *l*-sulfoxide and D-methionine *d*-sulfoxide and the other of a mixture of L-methionine *d*-sulfoxide and D-methionine *l*-sulfoxide. The sulfoxide isolated from the less soluble picrate decomposed at 233–234° and the sulfoxide from the more soluble picrate decomposed at 234–235° with much foaming, while the original mixture of the four stereoisomers melted with decomposition at 225–230°.

The present paper describes the oxidation of L-methionine by several agents including iodine, iodine in the presence of mercuric chloride (*i.e.* hypiodous acid), iodate, and hydrogen peroxide. Analytical evidence based on oxygen consumption and reduction values indicated that the product in each instance was methionine sulfoxide. However, discrepancies appeared in the decomposition points and optical rotations of the products and it was necessary to isolate and identify the sulfoxide with certainty, especially since "dehydromethionine" (Lavine (6)) is an intermediate in several of the oxidations and its decomposition might result in unknown products with similar properties. It was found, however, that the explanation of the results resided in the formation of varying amounts of the two diastereoisomeric sulfoxides of L-methionine; *i.e.*, the different methods of oxidation that produced the second center of asymmetry (the sulfoxide group) performed varying degrees of asymmetric synthesis, presumably as a result of varying rates of reaction. The two diastereo-

isomers, L-methionine *l*-sulfoxide and L-methionine *d*-sulfoxide, were best resolved by fractionation of the picrates. Elimination of the asymmetry of the carbon atom of these compounds by oxidation with chloramine-T resulted in products that initially possessed the rotation expected for the sulfoxide group. Some aspects of the rotatory properties of sulfur-containing amino acids are considered in the discussion.

Lampen et al. (3) and Waelsch and Borek (11) have demonstrated that the two diastereoisomeric sulfoxides derived from L-methionine exhibit different biological behavior and their findings may be expected to stimulate further investigations in this direction.

EXPERIMENTAL

The optical determinations were made with a Schmidt and Haensch polarimeter having 0.01° vernier divisions, with a Leitz sodium vapor lamp as a light source and either 1 or 2 dm. polarimeter tubes. The results are calculated in terms of the molar rotation, $[M]_D = [\alpha]_D \times \text{mol. wt. per 100}$. The L-methionine (Eastman Kodak Company) was used as received. Different lots assayed 96 to 98.7 per cent methionine according to iodometric determinations (Lavine (5)). The following rotations were obtained on 0.05 M solutions (corrected for methionine content): $[\alpha]_D^{24} = -7.9^\circ$ in water and $+24.5^\circ$ in N HCl. (The corresponding values for $[M]_D^{24}$ are -11.7° and $+36.5^\circ$.) The former value is in substantial agreement with reported values: -8.11° , Windus and Marvel (12); -7.9° , Pirie (7). Attention may be called to the difference in specific rotation of L-methionine in 0.5 M NaHCO₃ ($+12^\circ$) and borate buffer (-4.4°), although the pH was about 9.1 in both solutions.

Oxidation by Hydrogen Peroxide—This was carried out on 10 mm of L-methionine according to the procedure of Toennies and Kolb (9) for DL-methionine except that the oxidation was allowed to proceed for 3 hours instead of 0.5 hour; *i.e.*, after 0.5, 1, and 2 hours of oxidation, there remained respectively 10.4, 4.0, and 0.3 per cent of unoxidized methionine.¹ Yield, 98 per cent of the theory; decomposition point, 230°, marked by much foaming. The reduction value (*i.e.*, iodine liberated by treating a sample with 4 M HClO₄ and 2 M NaI for 2 hours under N₂ (9)), was 101.0 per cent of the theory for the sulfoxide. No methionine was present (5). The sulfoxide yielded the following molar rotations: $[M]_D^{25} = +58.8^\circ$ for

¹ 1 ml. of 2 N HCl, 1 ml. of 5 M KI, and 1 drop of 0.5 M ammonium molybdate were added to 0.5 ml. of the reaction solution, followed after a few seconds by the addition of a mixture of 2 ml. of 1 M NaOH and 1 ml. of 1 M phosphate buffer, pH 7. After standing about 3 minutes the excess iodine was titrated with thiosulfate and the value recorded as excess peroxide. The solution was then acidified by 2 ml. of 2 N HCl and the liberated iodine estimated by titration with thiosulfate and calculated as methionine (*cf.* Lavine (5)).

0.05 M solution in 1 M HCl; +24.8° for 0.1 M aqueous solution (pH 6.1); +33° for 0.05 M solution buffered at pH 7 (phosphate); +24.6° for 0.05 M solution buffered at pH 9 (borate); +19.4° for 0.05 M solution in 50 per cent methyl alcohol.

A second preparation obtained in 93.4 per cent yield provided the following data: decomposition point 226–228°; reduction value 101 per cent of the theory; amino N without KI, 109 per cent, with KI 104 and 106 per cent of the theory. The following molar rotations, $[M]_D^{25}$, were obtained: +25.7° for 0.167 M aqueous solution, +32° for 0.05 M solution at pH 7, +24° for 0.05 M solution at pH 9, and +59° for 0.05 M solution in N HCl.

Oxidation by Iodine—Oxidation of 0.05 M L-methionine in 0.2 M phosphate buffer at pH 7 by the theoretical amount of alcoholic iodine (0.05 M) in the presence of an equivalent amount of sodium hydroxide (0.10 M) occurred within 2 or 3 minutes, as evidenced by the disappearance of the iodine. The immediate oxidation product was not the sulfoxide, however, but an intermediate, "dehydromethionine" (Lavine (5, 6)), that is distinguished from the sulfoxide by its greater oxidizing power; *i.e.*, acidification of "dehydromethionine" to pH 0.5 to 1 in the presence of 1 M KI causes formation of a molar equivalent of iodine. The oxidizing power of the solution gradually decreased until after 165 hours only 1 per cent of the intermediate remained. The molar rotation, $[M]_D^{26}$, meanwhile decreased from +84° to +73°. The corresponding final value, $[M]_D^{26}$, in 1 N HCl was +107° (obtained on a solution prepared by adding 0.5 ml. of 10 N HCl and 3 drops of 0.025 N $\text{Na}_2\text{S}_2\text{O}_3$ to a 4 ml. aliquot and diluting to 5 ml.). Reduction of an aliquot (after 165 hours) by 4 M HClO_4 and 2 M NaI for 1 hour under CO_2 indicated 98.4 per cent of the calculated amount of sulfoxide.

Isolation of methionine sulfoxide after oxidation was carried out as follows: 5 mm of L-methionine were placed in a 50 ml. volumetric flask with 10 ml. of 1 M K_2HPO_4 and 14 ml. of water; 10 ml. of 0.447 M alcoholic I_2 were added, followed by 14.7 ml. of 0.409 M KOH (*i.e.* 6.0 mm of KOH in order to neutralize 60 per cent of the formed HI, leading to a final ratio of $\text{K}_2\text{HPO}_4:\text{KH}_2\text{PO}_4$ of 1.5 and a solution at pH 7). Additional iodine was added dropwise until the final drop slowly disappeared (total of 5.0 mm of I_2), after which the solution was diluted to volume and allowed to stand at room temperature. Table I illustrates the change in rotation accompanying sulfoxide formation. The data for the sulfoxide content are based on the disappearance of "dehydromethionine" (*i.e.*, 1 ml. of 5 M KI and 1 ml. of 2 N HCl were added to a ml. aliquot and the liberated iodine titrated with standard thiosulfate; the difference between this value and the total iodine added represents the sulfoxide present). Phos-

phate was precipitated from the residual solution by an equivalent amount of barium acetate (8.8 mm); there was no optical activity associated with the precipitated barium phosphate. The filtrate was evaporated to dryness and extracted with 1:1 water-methyl alcohol solution and acetone added until precipitation was complete. Considerable inorganic material was found to be present in the precipitate which was removed by solution of the material in 5 to 10 ml. of water and precipitation by 40 ml. of methyl alcohol. Yield, 41.5 per cent of the theory; on heating, the substance darkened and contracted at 236° but did not foam on heating to 283°. $[M]_D^{27} = +156^\circ$ for 0.05 M aqueous solution and $[M]_D^{26} = +188^\circ \pm 1^\circ$ for 0.05 M solution in 1 N HCl. Ultimate analysis yielded the following figures (Carl Tiedcke, New York).

$C_5H_{11}SNO_2$. Calculated.	C 36.36, H 6.715, N 8.48
Found.	" 36.55, " 6.67, " 8.69
	" 36.29, " 6.71, " 8.57

TABLE I
Sulfoxide Formation Following Oxidation of Methionine by Iodine at pH 7

Time, hrs.	0.25	1	20	44	68	140
$[M]_D^{25}$, degrees		+85.5	+81.7	+77	+75.5	+72
Sulfoxide, %	6.2	10.0	60.9	82.9	91.4	98.8

Decomposition of Intermediate Oxidation Product by Acid—If, after iodometric oxidation of methionine at pH 7, iodide is removed as silver iodide and the solution then made 1 M in HCl, the intermediate is found to be 78 and 94 per cent decomposed 5 and 15 minutes respectively after the addition of acid. A 0.05 M solution in 1 N HCl prepared in this way, with silver perchlorate to precipitate iodide, was found to have a molar rotation, $[M]_D^{25}$, of $+86^\circ$. Identification of the reaction product as methionine sulfoxide was established by the following evidence obtained on the product isolated in 80 per cent yield after oxidation of DL-methionine: reduction value, 100.4 per cent of the theory; equivalent weight by titration with acetous perchloric acid (Toennies and Callan (8)), 165.4; theory 165.1; decomposition point 226–227°; there was no oxidation by iodine at pH 7 or by iodate in N HCl over a period of 1 hour.

Oxidation by Iodine in N HCl in Presence of Mercuric Chloride—Methionine is oxidized very slowly by iodine in N HCl, but when mercuric chloride is also present 1 mole of iodine is consumed within 10 minutes. The evidence for sulfoxide formation is based on the quantitative aspects of iodine consumption and on the irreversibility of the oxidation. The oxidation of 0.05 M L-methionine in 1 N HCl and 0.2 M mercuric chloride

by 0.05 M iodine led to a solution (after filtering from mercuric iodide) having $[M]_D^{25} = +86^\circ$ that was constant for 1 hour.

Iodate in N HCl—Methionine (0.01 M) is rapidly oxidized by iodate (0.05 N) in N HCl with 99.6 and 100.2 per cent sulfoxide formation after 10 and 60 minutes, respectively. On further standing, the oxidation continued and a value 144.0 per cent of the theory was obtained after 18 hours. 15 minutes after preparation, a solution 0.05 M in L-methionine, 0.15 N in KIO_3 , and 1 N in HCl possessed $[M]_D^{25} = +73^\circ$; after 21 hours $[M]_D^{26} = +71.4^\circ$.

The results of these oxidations are summarized in Table II. Since the sulfoxide was produced in all cases, it is evident from the rotations that

TABLE II
Asymmetric Oxidation of L-Methionine to Sulfoxide

Solution No.	Method of oxidation	$[M]_D^{25-26}$ in N HCl	Per cent* L-methionine d-sulfoxide	Time for complete oxidation to sulfoxide
		<i>degrees</i>		
1	H ₂ O ₂ in 0.46 N HCl	+59	50.6	2 hrs.
2	KIO ₃ in N HCl	+73	55.1	5-10 min.
3	I ₂ and HgCl ₂ in N HCl	+85	59.0	< 10 min.
4	I ₂ at pH 7 followed by acidification (N HCl)	+86	59.4	2 to 3 min. for I ₂ oxidation; about 15 min. for sulfoxide formation
5	I ₂ at pH 7; let stand at pH 7	+107	66.2	2 to 3 min. for I ₂ oxidation; about 150 hrs. for sulfoxide formation
	Isolated material	+188	92.8	

* $100(95.2 + [M]_D)/305.2$ (cf. "Resolution of diastereoisomers").

the proportion of the two diastereoisomeric sulfoxides must have differed in the various products, and further that the more rapid the reaction the greater was the degree of asymmetric synthesis. Little can be said concerning the secondary reactions of Solution 5, but a series of consecutive reactions is indicated, of which the asymmetric step must be rapid despite the slow over-all rate of reaction. The similarity of the rotations of Solutions 3 and 4 suggests that the mechanism of the reaction and the intermediate involved were the same in these two cases. When L-methionine (0.05 M) was oxidized by peroxide under conditions leading to the sulfone (Toennies and Kolb (10)), i.e. by 0.15 M H₂O₂, 0.02 M (NH₄)₂MoO₄ in 1 M HCl, $[M]_D^{25} = +65.5^\circ$, $+56.5^\circ$, $+54.5^\circ$, and $+49^\circ$ after 0.41, 1.7, 2, and 22 hours, respectively. The final value presumably represents the

sulfone derived from L-methionine, but the high initial rotation compared with that produced by hydrogen peroxide alone (Solution 1) indicates that molybdate caused a greater degree of asymmetric synthesis as well as more rapid reaction.

No evidence of racemization or epimerization was noted. The rotations of the product isolated after H_2O_2 oxidation were constant for 7 days in both N HCl and in water ($[\text{M}]_D = +59^\circ$ and $+27^\circ$, respectively). Similar results were obtained over a period of 4 days with the product possessing $[\text{M}]_D = +188^\circ$ in N HCl and $+156^\circ$ in water. Oxidation by hydrogen peroxide (Solution 1) results in a product that contains almost equimolar amounts of the two diastereoisomers and, for certain purposes, hereafter, this substance will be employed and designated as L-methionine *dl*-sulfoxide.

Resolution of Diastereoisomers—Since the two sulfoxides are diastereoisomers, resolution by physical methods should be possible. The isolation of a product with a molar rotation of $+188^\circ$ from a reaction solution (Solution 5, Table II) with a rotation of $+107^\circ$ suggests that recrystallization from water-methyl alcohol mixtures might easily effect such a separation. However, when the sulfoxide was subjected to fractional crystallization by gradually increasing the methyl alcohol content of an aqueous solution, it soon became apparent that a sharp separation would require many fractionations.² Accordingly, resort was had to fractionation of the picrates, a technique employed by Toennies and Kolb (unpublished) that apparently sharply resolved DL-methionine *dl*-sulfoxide into two racemic mixtures.

In this procedure, equimolar amounts of solid picric acid and methionine sulfoxide were heated on a boiling water bath with the minimal amount of water necessary for solution. (4 ml. of water were sufficient to dissolve the picrate from 1 gm. of DL-methionine *dl*-sulfoxide at 96° .) On cooling, a crystalline picrate was deposited that was filtered off. A second fraction was obtained by evaporating the filtrate to incipient crystallization, cooling, and washing the resulting residue with acetone.

The two picrates were decomposed by adding 10 to 25 per cent excess amylamine to an aqueous solution and precipitating the sulfoxide with acetone.

² 1.55 gm. of methionine sulfoxide with a molar rotation of $+25.2^\circ$ (0.05 M in aqueous solution) was recrystallized from 40 ml. of 80 per cent methyl alcohol; yield, 82 per cent; $[\text{M}]_D = +38^\circ$. The latter material (1.27 gm.) was dissolved in 10 ml. of water and the solution made successively 60, 75, 85, and 91 per cent in methyl alcohol by addition of methyl alcohol to the filtrate from each precipitate. The molar rotations of the corresponding fractions (aqueous 0.05 M solutions) were $+123^\circ$, $+98^\circ$, $+11^\circ$, and -6° , respectively. The levorotatory sulfoxide is accordingly the more soluble.

Application of this procedure to L-methionine *dl*-sulfoxide showed one of the picrates to be sparingly soluble (17 ml. of water at 96° did not dissolve the picrate from 1 gm. of sulfoxide). The picrate (from 1.33 gm. of sulfoxide) was accordingly filtered off after the solution was cooled to room temperature, washed with water, and decomposed with amylamine without recrystallization. A white crystalline product was obtained (0.615 gm.) that possessed molar rotations, $[M]_D^{25}$, as follows: +138° for 0.05 M aqueous solution and +183.4° for 0.05 M solution in N HCl. The substance sintered at 250–255° after gradually darkening, but there was no foaming on heating to 275°.

The mother liquor containing the soluble picrate was evaporated at room temperature *in vacuo* nearly to dryness, diluted with water to a volume of about 5 ml., and filtered with 2 ml. of water for rinsing and washing. (In view of later results it would appear that a supersaturated solution of the picrate was formed.) 10 ml. of methyl alcohol and 4 mm of amylamine were added to the filtrate, followed by the addition of 75 ml. of acetone. The sulfoxide was filtered, washed with acetone, and dried; yield, 0.515 gm. This fraction possessed a molar rotation, $[M]_D^{25}$, of –110.2° in water and –86.6° in 1 M HCl (0.05 M solutions). On heating at the rate of 2° per minute, the substance gradually darkened, contracted at 235°, and decomposed with gas evolution and foaming at 239°.

It is evident from the results of Table II that complete resolution, at least of the dextrorotatory sulfoxide, was not achieved and it appeared desirable to modify the procedure somewhat in order to eliminate the opportunity for occlusion presented by the low solubility of the dextrorotatory picrate. The slow addition of a picric acid solution to the sulfoxide should avoid this difficulty and also might selectively precipitate the dextrorotatory picrate. Accordingly, 11.7 ml. of 0.641 M picric acid (7.5 mm) in methyl alcohol were slowly added to 14.3 mm of methionine sulfoxide³ dissolved in 10 ml. of water. After a short time, a precipitate was formed that amounted to about 45 per cent of the theory. However, the solubility of this material when washed with water indicated it to be a mixture of the picrates and it was recombined with the solution. An additional 7.5 mm of alcoholic 0.641 M picric acid were added, making the total volume of the solution about 100 ml. After standing overnight, the precipitate was filtered off, washed with water, and treated according to the following procedure: 30 ml. of water were added, and the mixture heated at 70–75° for 10 minutes and then let stand for 23 hours at room

³ This product was prepared by hydrogen peroxide oxidation for only 1 hour and was later found to contain 4.6 per cent methionine. $[M]_D^{20} = +64.7^\circ$ in N HCl and +29.8° in water (corrected for methionine content). The methionine was removed, as shown in the text, in the filtrate from the levorotatory picrate.

temperature; the precipitate was again removed and the filtrate analyzed (1) for picric acid by titration with 0.05 N NaOH with bromocresol purple as indicator, (2) for sulfoxide by titration of the iodine liberated after treatment with 4 M HClO₄ and 2 M NaI for 2 or 3 hours under nitrogen, and (3) for its rotation. This procedure was repeated on the washed precipitate until the molar rotation of the filtrate became constant within the limits of the methods as shown in Table III. The final value was checked by the rotation of the precipitate. The picrate (1.25 gm.) was then decomposed by adding 20 ml. of water and about 10 per cent excess amylamine (or until alkaline to litmus); the sulfoxide was precipitated by 200 ml. of acetone. After the precipitated sulfoxide had settled clear, it was filtered off and washed with acetone until all of the amylamine picrate was

TABLE III
Fractionation of Dextrorotatory Picrate of L-Methionine Sulfoxide

Solution No.	Volume	Picric acid	Sulfoxide	α per dm	$[M]_D^{25}$
	ml.	M	M	degrees	degrees
1	30	0.0243	0.0243	-0.052	-21
2	30	0.0184	0.0184	+0.087	+47
3	30	0.0180	0.0185	+0.093	+50
4	30	0.0159	0.0155	+0.210	+136
5	30	0.0156	0.0153	+0.245	+160
6	12	0.0135	0.0134	+0.241	+179 \pm 5
7	18	0.0134	0.0129	+0.247	+187 \pm 5
0.01 M solution of ppt.		0.0099	0.0104	+0.180	+180 \pm 6

removed; yield, 0.50 gm. This product was characterized as follows: $[M]_D^{25} = +163.5^\circ$ ($[\alpha]_D^{25} = +99^\circ$) for 0.1 M aqueous solution; $[M]_D^{25} = +210^\circ$ ($[\alpha]_D^{25} = +127^\circ$) for 0.05 M solution in 1 N HCl; on heating, the substance gradually darkened and sintered at 240-245°. No methionine was present in this material and 100.4 per cent of the theoretical amount of iodine was liberated on reduction by 4 M HClO₄ and 2 M NaI for 2 hours under nitrogen.

It was subsequently found that an extraction with 25 ml. of water, carried out as previously outlined (filtrate, $[M]_D = -41^\circ$, 0.04 M), followed by a similar treatment with 25 ml. of methyl alcohol (filtrate, $[M]_D = +123^\circ$, 0.011 M) sufficed for optical purification of 1.98 gm. of the dextrorotatory picrate (final value of $[M]_D = +182^\circ$).

The original filtrate (100 ml.) from the dextrorotatory picrate was evaporated to about 25 ml. on a water bath (1 hour) and finally to dryness at room temperature. The residue was stirred with 33 ml. of water,

filtered at once, and the filtrate discarded.⁴ The remaining precipitate, except for 0.087 gm. of insoluble material, was dissolved in 150 ml. of water by washing on a sintered glass funnel. The resulting solution was again evaporated to dryness and the residue stirred with 5 ml. of water, which caused the formation of a turgid crystalline mass. An additional 10 ml. of water were added and the mixture was heated on the water bath, thereby effecting nearly complete solution of the precipitate. After standing overnight at room temperature the precipitate and filtrate were

TABLE IV
Fractionation of Levorotatory Picrate of L-Methionine Sulfoxide

	Picric acid	Sulfoxide	α per dm.	$[M]_D^{25-20}$
	<i>M</i>	<i>M</i>	degrees	degrees
1. Ppt., 1.98 gm.	0.0255	0.0249	-0.196	-78.1
Filtrate, 20 ml.	0.0322	0.0170	-0.113	-66.4
2. Ppt., 1.88 gm. heated with 15 ml. water; filtered hot from a slight residue, and let stand overnight at room temperature; filtered				
Ppt., 1.32 gm.	0.0250	0.0251	-0.195	-78.2
Filtrate	0.0565	0.0514	-0.305	-59.4
3. Ppt., 1.22 gm., heated with 25 ml. methyl alcohol, almost all dissolved; let stand overnight at room temperature; filtered				
Ppt., 0.77 gm.	0.0250	0.0250	-0.266	-106.5
Filtrate	0.0549		-0.180	-32.7
4. Ppt., 0.66 gm., heated with 15 ml. methyl alcohol; let stand overnight at room temperature; filtered				
Ppt., 0.37 gm.	0.0250	0.0250	-0.265	-106
" (methyl alcohol)	0.0250	0.0250	-0.225	-89
Filtrate	0.0485		-0.407	-84

The figures in bold-faced type represent concentrations calculated from a weighed sample.

separately analyzed as shown in step (1) of Table IV. A second similar treatment (step (2)) removed considerable material without effecting any improvement in the optical rotation of the precipitate. Since the dextro-rotatory picrate was found to be sparingly soluble in methyl alcohol (a saturated solution at 25° was 0.0075 M with $[M]_D^{25} = +185^\circ$) the precipitate was treated with methyl alcohol as shown by step (3). The results were surprising in this case as well as in the preceding steps in that the filtrate contained the excess of the dextrorotatory picrate, indicating that

⁴ The solution after partial evaporation at room temperature deposited 0.04 gm. of material ($[M]_D = +13^\circ$) and left a solution containing a total of 0.44 mm of sulfoxide, 1.6 mm of picric acid, and 0.6 mm of methionine; α per dm. was -0.048° ; volume 25 ml.

the solubility of the latter is increased in solutions of the levorotatory picrate. Repetition of the extraction with methyl alcohol (step (4)) indicated that the precipitate and filtrate were optically uniform. The precipitate and filtrate of step (4) and the rotation solutions were combined (total of 1.9 mm of picrate) and evaporated to about 40 ml. The picrate was decomposed by 0.26 ml. of 8.23 M amylamine and the sulfoxide precipitated by acetone. Yield, 0.248 gm. (1.5 mm); $[M]_D^{26} = -118.4^\circ$ for 0.05 M aqueous solution and -95.2° for 0.05 M solution in 1 N HCl (the corresponding specific rotations, $[\alpha]_D^{26}$, are -71.6° in water and -57.6° in N HCl); the decomposition point was $238-239^\circ$ after contraction at 233° . The decomposition of this substance was usually marked by much foaming up the capillary. No methionine was present in this material and reduction by HClO_4 and NaI for 2 hours yielded 100.1 per cent of the theoretical value.

The two diastereoisomers may be designated L-methionine *d*-sulfoxide and L-methionine *l*-sulfoxide, in which the second prefix indicates the direction of rotation of the sulfoxide group. Qualitatively, L-methionine *l*-sulfoxide was found to be appreciably soluble in methyl alcohol while L-methionine *d*-sulfoxide was almost insoluble.

The percentages of this last isomer present in the various solutions listed in Table II were calculated by use of the equation, $x = (100(95.2 + [M]_D^{26})) / (95.2 + 210)$, which is derived from the molar rotations of the unknown and of the two diastereoisomers in N HCl.

Elimination of Asymmetry of α -Carbon Atom—Oxidation of an α -amino acid by chloramine-T proceeds at room temperature and results in the formation of ammonia, carbon dioxide, and an aldehyde or nitrile, depending on conditions (Dakin (1), Friedman and Morgulis (2)). In the application of this reaction to methionine sulfoxide, equimolar amounts of the two substances were employed in order to restrict the possibility of oxidation of the sulfoxide group and, accordingly, formation of the aldehyde, β -methylsulfinylpropionaldehyde, may be expected.

In 40 per cent methyl alcohol the reaction between equimolar amounts of chloramine-T and methionine sulfoxide (0.05 to 0.10 M) was 97 per cent complete in 7 minutes and finished within 12 minutes as shown by the disappearance of chloramine-T (*i.e.* by the iodine liberated from KI at pH 7). In aqueous solution about 1 hour was necessary for disappearance of chloramine-T. The *p*-toluenesulfonamide was soluble in 40 per cent methyl alcohol but started precipitating immediately from the aqueous solution. When the L-methionine *dl*-sulfoxide (0.05 M) was oxidized by a molar equivalent of chloramine-T in 40 per cent methyl alcohol, the molar rotation decreased from $+24.8^\circ$ to $+3^\circ$ (final α per dm. = $+0.014^\circ$ to $+0.02^\circ$) in 10 minutes and remained constant for 24 hours. With a second prepara-

tion (0.05 M), the rotation decreased from $+26^\circ$ to $+4^\circ$. These results indicate that the asymmetry of the carbon atom was destroyed and the residual rotation may be attributed to the slight excess of L-methionine *d*-sulfoxide present in the starting material. When L-methionine *d*-sulfoxide (0.05 M) and L-methionine *l*-sulfoxide (0.025 M) were treated similarly with chloramine-T in 40 per cent methyl alcohol solution, the respective rotations changed in 10 minutes from $+163.5^\circ$ to $+145^\circ$ and from -118.4° to -142° . The values did not remain constant, however, and continued

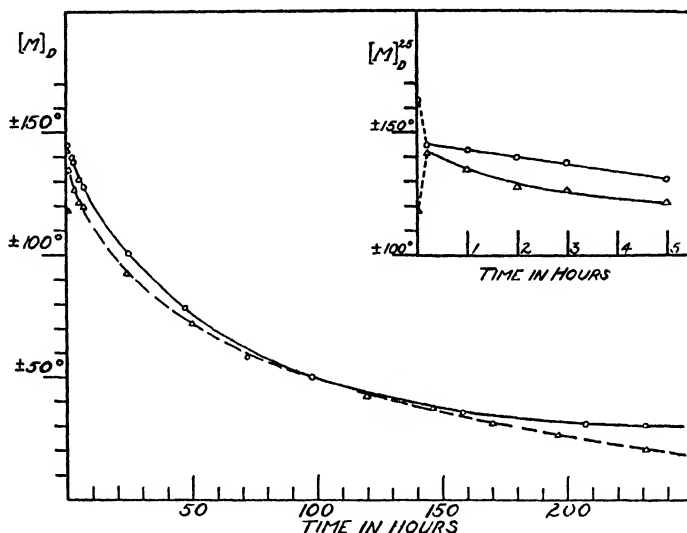


FIG. 1. Change in rotation after reaction of methionine sulfoxide with a molar equivalent of chloramine-T in 40 per cent methyl alcohol. \circ , dextrorotatory values for 0.05 M L-methionine *d*-sulfoxide; $[M]_D$ after 326 hours = $+30^\circ$; \triangle , levorotatory values for 0.025 M L-methionine *l*-sulfoxide; $[M]_D$ after 289 hours = -14° . The temperature was 23–25°. The inset shows the rotations for the first 5 hours.

to change as shown by Fig. 1. It should be noted that the limiting value of the rotation was not always zero. Further data are required before a decision can be made as to the nature of the reaction responsible for the continued decrease in rotation. The reduction values for the sulfoxide group were 95 to 100 per cent of the theory immediately after reaction with chloramine-T, indicating that this group was not attacked. After 2 weeks, however, the reduction values also decreased, but in rather erratic fashion, to 53, 60, and 74 per cent of the theory for L-methionine *dl*-sulfoxide, L-methionine *d*-sulfoxide, and L-methionine *l*-sulfoxide, respectively. The formation of a product resistant to reduction was indicated by finding that the values were increased about 28 per cent when the reduction was

allowed to continue overnight instead of 3 hours. The determination of aldehyde, by estimation of excess bisulfite after an aliquot had remained for 1 hour with a measured excess of bisulfite, yielded values amounting to 50 to 60 per cent of the theory; estimation of bound bisulfite after destruction of the aldehyde-bisulfite by bicarbonate generally yielded lower values.⁵ Precipitation by 2,4-dinitrophenylhydrazine also indicated the presence of an aldehyde; yield, 37 per cent, m.p. 137–140°.

DISCUSSION

One-half the algebraic sum of the rotations of the two diastereoisomers results in values of +22.6° in water and +57.4° in *N* HCl for the molar rotations of L-methionine *dl*-sulfoxide, which also represent the contribution of the asymmetric carbon atom to the total rotation of L-methionine sulfoxide. Deduction of these values for carbon from the rotations of the two diastereoisomers leads to $\pm 141^\circ$ in water and $\pm 152.6^\circ$ in *N* HCl as the molar rotation of the sulfoxide group in methionine sulfoxide. The action of chloramine-T on the L-methionine *dl*-, the L-methionine *d*-, and the L-methionine *l*-sulfoxides resulted in decreases of dextrorotation of 22°, 18.5°, and 23.6°, respectively, that are consistent with the calculated values for carbon. The L-methionine *d*- and L-methionine *l*-sulfoxides yielded initial values of +145° and -142° that are in good agreement with the calculated values for the sulfoxide group. The latter results also suggest that the rotation of the sulfoxide group is but slightly affected by changes in structure about the asymmetric carbon atom. In other words, although quantitative formation of the aldehyde may be questioned, the evidence indicates that the asymmetry of the carbon was destroyed and, as far as the rotation of the sulfur is concerned, it appears of little consequence whether the γ position to the sulfur is occupied by $-\text{CH}(\text{NH}_2)\text{-COOH}$, $-\text{CHO}$, or intermediate structures. Further evidence in this direction is supplied by finding that the following changes in the medium affected the rotation of only the carbon atom: the rotation of L-methionine *d*-sulfoxide was +172° in a solution buffered at pH 7 and +157.6° in 50 per cent methyl alcohol, which differ from the rotation in aqueous solution (+163.5°) by +8.5° and -5.9°, respectively. Since essentially the same differences, +8.2° and -5.4°, were obtained for corresponding solutions of L-methionine *dl*-sulfoxide (*cf.* "Oxidation by hydrogen peroxide"), it is evident that only the rotation of the carbon atom was altered. The 8.5 per cent increase in rotation of the sulfoxide group in *N* HCl over that in water probably reflects the slight basic properties of the sulfoxide group.

⁵ In titrating bisulfite in bicarbonate solution with iodine, it was necessary to make the solution 1 *M* in KI in order to prevent side oxidations.

The rotatory power of the asymmetric carbon atom on the other hand is influenced considerably by changes in the sulfur atom. The conversion of L-methionine to its *dl*-sulfoxide changes the molar rotatory power of the carbon atom from -12° to $+23^\circ$ in aqueous solution and from $+36.5^\circ$ to $+57^\circ$ in *N* HCl. Further evidence of the effect of the state of sulfur on the asymmetric carbon atom is furnished by the large increase in rotation that is obtained when L-methionine is dissolved in 1 *N* HCl and 1 *M* HgCl_2 ($[\text{M}]_D^{25} = +79^\circ$). That the sulfur atom is responsible for the increased rotation may be concluded from the unaltered rotations of 0.025 *M* L-methionine *d*-sulfoxide ($[\text{M}]_D^{24} = +211.6^\circ$) and 0.025 *M* L-methionine *dl*-sulfoxide ($[\text{M}]_D^{24} = +59.6^\circ$) in *N* HCl and 0.5 *M* HgCl_2 . This behavior of methionine is similar to the increase in molar rotation ($[\text{M}]_{\text{Hg}}^{24}$) from $+5^\circ$ to $+123^\circ$, that results when a 0.01 *M* solution of L-cysteine in 1 *N* HCl is made 0.05 *M* in HgCl_2 (Lavine (4)); on the other hand, the rotations of cystine, cysteinesulfinic acid, and cysteic acid are unaffected by the presence of mercuric chloride, which demonstrates that in *N* HCl solution the amino group is not involved in this phenomenon. The increased rotation in the presence of mercuric chloride may be due, especially in the case of methionine, to the formation of an asymmetric complex with the sulfur atom analogous to a sulfonium salt, or to the fact that formation of a mercury complex induces a polarity on the sulfur atom that directly or indirectly affects the asymmetric carbon atom. With respect to this latter factor, the great increase in optical rotation that accompanies disulfide formation from mercaptans like cysteine, glutathione, etc., may be related to the polarity of the disulfide linkage, $\text{RS}^+ - \text{S}^- \text{R} \rightleftharpoons \text{RS}^- - \text{S}^+ \text{R}$, and its effect on the asymmetric carbon atom.⁶

⁶ Comparison of the specific rotation, $[\alpha]_{\text{Hg}}^{25}$ of *p*-tolylalanyl disulfide (-250°) with that of cystine (-253°) is suggestive of a fixed polarity in the disulfide linkage of the former compound, since the rotation of its half cystine residue is about twice that of a half cystine residue in cystine. The rotations were taken on 0.05 *M* solutions in 0.5 *M* HCl in aqueous ethanol, 50 per cent by volume. *p*-Tolylalanyl disulfide was prepared according to the reaction, $\text{RSO}_2\text{SR} + \text{ArSH} \rightarrow \text{ArSSR} + \text{RSO}_2\text{H}$, from L-cystine disulfoxide and *p*-thiocresol as follows: 5 mm of *p*-thiocresol were added to 5 mm of L-cystine disulfoxide dissolved in 5 ml. of 5 *M* HCl and the mixture diluted to 50 ml. with 95 per cent ethanol. After standing 2 hours at room temperature, the solution was neutralized with 2.3 ml. of pyridine and allowed to stand overnight. The precipitate of thin, glistening plates of irregular, hexagonal shape was filtered by suction and washed with ethanol and ether. The filtrate contained 98.6 per cent of the calculated amount of cysteinesulfinic acid, based on iodine liberation in 1 *M* HCl and 1 *M* KI. The precipitate amounted to 1.00 gm. or 82 per cent of the theory; sulfur content 26.4 per cent, theory 26.37 per cent; m. p. $185-186^\circ$ with decomposition, unchanged by reprecipitation from aqueous ethanolic HCl solution. The substance is odorless, tasteless, almost insoluble in water or ethanol, and sparingly soluble in 2 *N* HCl. $[\alpha]_{\text{Hg}}^{26.5} = -208.4^\circ$ for an 0.05 *M* solution in ethanol that contained 19 per

The author is indebted to G. Toennies and J. J. Kolb for the unpublished data on the partial resolution of DL-methionine sulfoxide by picric acid, and to Carlota Herkness for technical assistance.

SUMMARY

The following methods of oxidation of L-methionine were investigated: (1) hydrogen peroxide, (2) iodate in N HCl, (3) iodine in N HCl in the presence of mercuric chloride, and (4) iodine at pH 7; the latter procedure resulted in an intermediate oxidation product that was decomposed (a) in N HCl and (b) in neutral solution. γ -Methylsulfinyl- α -aminobutyric acid, for convenience called methionine sulfoxide, was the final oxidation product in all cases. Since the sulfoxide group constitutes a second center of asymmetry in the molecule, the product contained two diastereoisomers that were designated L-methionine *d*-sulfoxide and L-methionine *l*-sulfoxide. The rate of reaction and the relative amount of the first of these two isomers were found to increase according to the above sequence of reactions; hydrogen peroxide oxidation yields almost equal amounts of the two isomers, *i.e.* L-methionine *dl*-sulfoxide.

The two diastereoisomers were resolved as picrates. L-Methionine *d*-sulfoxide forms a sparingly soluble picrate, possesses a molar rotation, $[M]_D^{25}$, of $+163.5^\circ$ in aqueous solution and $+210^\circ$ in N HCl ($[\alpha]_D^{25} = +99^\circ$ and $+127^\circ$, respectively), and does not have a well defined decomposition point. L-Methionine *l*-sulfoxide forms a more soluble picrate, possesses a molar rotation, $[M]_D^{25}$, of -118.4° in water and -95.2° in N HCl ($[\alpha]_D^{25} = -71.6^\circ$ and -57.6° , respectively), and decomposes at $238-239^\circ$, usually with much foaming.

Reaction of the two diastereoisomeric sulfoxides with chloramine-T led to solutions that initially possessed rotations calculated for the sulfoxide group. The elimination of the asymmetry of the carbon atom was indicated, but the nature of the resulting molecule was not definitely established, although evidence for the formation of an aldehyde, presumably β -methylsulfinylpropionaldehyde, was obtained.

Factors influencing the rotatory power of the two asymmetric centers were discussed.

cent water by volume and was 0.5 M in HCl. Reduction in aqueous alcoholic HCl solution by zinc dust resulted in formation of cysteine and *p*-thiocresol (identified by odor and solubility characteristics) to the extent of 99.6 per cent of the theory on the basis of subsequent iodometric oxidation in 1 N HCl and 1 M KI to the respective disulfides. The presence of the *p*-thiocresyl radical was also indicated by the formation of a blue color on warming the substance in concentrated sulfuric acid. Oxidation by iodate in N HCl was slow compared with cystine but yielded values after 24 and 72 hours, amounting respectively to 98.4 and 99.4 per cent of the theory for oxidation to cysteic and *p*-toluenesulfonic acids.

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GLYCOLYSIS IN HUMAN BLOOD

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The prospect of studying human leucemic blood by means of the Warburg techniques raised the question of the glycolytic rate in normal human blood. The pioneer work in this field was that of Evans (1) who demonstrated that the fall in the CO_2 capacity of shed blood was due to conversion of glucose to lactic acid. The topic of glycolysis was reviewed by Lunds-gaard in 1933 (2). Although the effect of change in pH on glycolysis is marked (3), few attempts were made to control CO_2 tension in much of the earlier work. When manometric methods were used, the evolution of CO_2 was assumed to be due to lactic acid formation, and manometric data were frequently not correlated with chemical analyses. Further, without the benefit of chemical analyses, the assumption has been made that glucose utilization equals lactic acid formation. Lastly, there is considerable variation as to the glycolytic rate in different reports. This is illustrated by Table I, which presents a partial tabulation of reports on anaerobic and aerobic glycolysis in the peripheral blood of man. The glycolytic rate, symbolized by Q_G^O for aerobic glycolysis and Q_G^N for anaerobic glycolysis, is defined as the number of c.mm. of CO_2 produced by, or equivalent to, the acid formation per mg. of cell protein per hour. The data of Table I have been recalculated when necessary on this basis with our value of 158 mg. for the weight of total cell protein per 1 ml. of whole blood.

The work to be reported here was undertaken in an attempt to answer the following questions. Can the glycolytic rate in whole human blood be precisely defined? What is the quantitative relationship of Q_G^N to Q_G^O ? What is the correlation between the evolution of CO_2 determined manometrically and the chemical analyses for lactic acid and glucose?

EXPERIMENTAL

Methods

The whole venous blood of healthy male and female laboratory workers was used. Counts for red and white blood cells were performed on each sample. The anticoagulant was heparin. To determine whether or

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not the initial glucose concentration influenced glycolysis, about half of the blood samples were fortified by the addition of approximately 100 mg. per cent of glucose when drawn. No differences were noted between fortified and unfortified samples.

Manometric determinations were made with conventional Barcroft-Warburg vessels having a total capacity of 8 ml. Each vessel contained 2 ml. of whole heparinized blood. An equilibration period of 15 minutes was allowed to establish aerobic or anaerobic conditions as required. The particular gas mixture desired (95 per cent O_2 -5 per cent CO_2 for aerobiosis, 95 per cent N_2 -5 per cent CO_2 for anaerobiosis) flowed rapidly through the vessels for the duration of the equilibration period. During this time the vessels were immersed in the water bath and were shaken at a rate of approximately 120 strokes per minute. Equilibration of the

TABLE I
Representative Values for Anaerobic and Aerobic Glycolysis in Human Blood

Material	No. of experiments	$Q_G^{N_2}$	$Q_G^{O_2}$	Author
Red blood cells less buffy coat, defibrinated serum	3	+0.27		Burger (4)
Washed cells, whole blood, Ringer-glucose	8		+0.25	Engelhardt and Ljubimowa (5)
Whole blood, defibrinated			+0.27	Somogyi (6)
" " oxalated	10		+0.10	Katayama and Garcia (7)
" " "	8		+0.13	" (8)
" " heparinized	17		+0.30	Schmitz and Glover (9)

blood samples with a physiological tension of carbon dioxide as described here is essential in experiments of this nature, since only in this way can the pH of the blood be restored to that existing at the time of venipuncture and be maintained reasonably constant during an experiment.

After the gas flow was discontinued and the vessels closed off, a 5 minute interval was allowed for temperature and pressure equilibration before beginning manometric readings. The experimental period was either 60 or 180 minutes. Manometric readings of CO_2 evolution were corrected for retention by the plasma as described by Dixon (10) and by Umbreit *et al.* (11).

The total contents of each vessel were analyzed for glucose and lactic acid. These analyses were run on the contents of vessels removed from the water bath at zero time of the manometric experimental period and on the contents of others removed at the end of the experiment. Such analyses are referred to as initial and final glucose and lactic acid levels respec-

tively. In each volume of blood, 1 volume of 10 per cent sodium tungstate and 1 volume of 0.66 N sulfuric acid were used for the precipitation of protein at a final dilution of 1:12.5. Blood sugar was determined by the method of Benedict (12) in the Klett-Summerson photoelectric colorimeter. Lactic acid was determined according to the method of Barker and Summerson (13). The analytical accuracy of each series of experiments was controlled by the analysis of a separate control sample of blood to which known amounts of glucose and lactic acid had been added.

Kjeldahl determinations for the total nitrogen of the washed cells of several 1 ml. samples of whole blood were made. These determinations were corrected on the basis of cell counts so as to represent the total nitrogen of the washed cells of 1 ml. of whole blood having an erythrocyte count of 5×10^6 per c.mm. An average figure of 25.3 mg. of total cell N was obtained. From this it was calculated, with the customary factor of 6.25, that the cells of 1 ml. of whole blood contained 158 mg. of total cell protein.

Results

Thirty-eight experiments were performed. In the first ten the experimental period was 180 minutes. In each of these early experiments the evolution of gas during the first 60 minutes was 2 to 6 times as great anaerobically as aerobically. In the subsequent 120 minutes more gas was evolved by the blood under anaerobic conditions, but the difference between anaerobiosis and aerobiosis was small. Thus for the first, second, and third 60 minute periods the average anaerobic gas evolution per 1 ml. of blood was +174, +69, +61 c.mm., respectively, and the average aerobic gas evolution was +50, +38, and +38 c.mm. Since chemical analyses gave no support for such an enhanced glycolytic rate during the 1st hour of anaerobiosis, an alternate explanation for this phenomenon was sought. It appeared likely that the 15 minute equilibration period with the N_2 - CO_2 gas mixture for the establishment of anaerobiosis was insufficient for the complete dissociation of O_2 from hemoglobin, and that subsequent liberation of O_2 might account for the apparently excessive evolution of gas during the 1st hour. This was confirmed by the demonstration in the Summerson differential manometer (14) that the bulk of the gas liberated in the anaerobic vessel during the 1st hour was not absorbed by alkali. Consequently, the equilibration with 95 per cent N_2 -5 per cent CO_2 for only 15 minutes was not suitable for the manometric determination of anaerobic glycolysis in whole blood. Since carbon monoxide is reported not to affect glycolysis (8), it was decided to equilibrate the blood to be used in the anaerobic experiments with 100 per cent CO for 10 minutes prior to the 15 minutes equilibration with the N_2 - CO_2 mixture. The effect of such preliminary equilibration with CO on the manometric results during

the 1st hour of anaerobiosis, as well as the relation between anaerobic and aerobic readings under these conditions, is illustrated in Fig. 1. In all subsequent experiments, including those reported in Table II, anaerobiosis was obtained by first exposing the blood to an atmosphere of nearly

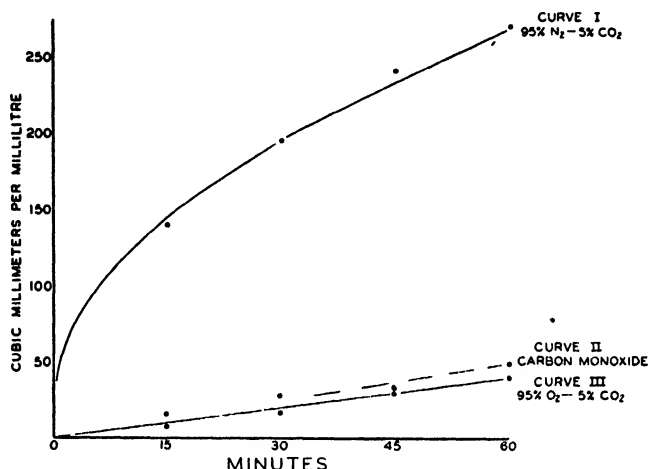


FIG. 1. Effect of preliminary equilibration with carbon monoxide on the manometric determination of anaerobic glycolysis.

TABLE II
Leucocyte and Erythrocyte Counts of Blood Used in Each Experiment

Experiment No.	Red blood cells, 10^6	White blood cells, 10^3	Experiment No.	Red blood cells, 10^6	White blood cells, 10^3
20	5.0	8.0	33	6.2	12.9
21	5.2	5.6	34	5.5	12.2
22	5.6	5.1	35	4.6	4.7
27	4.7	6.6	36	4.6	9.3
28	5.0	8.5	36B	5.2	5.3
29	4.7	5.5	37	4.7	6.9
30	5.6	13.1	38	5.2	8.4
31	5.6	6.9	39	4.8	10.5
32	5.6	9.6	40	5.1	10.7

pure CO for 10 minutes followed by the routine N₂-CO₂ equilibration for 15 minutes.

The results, which are considered valid, of eighteen experiments are reported in detail in Tables II, III, and IV and Fig. 2. Table II gives the leucocyte and erythrocyte counts of the blood used in each experiment. Table III presents the manometric and chemical data as observed in

TABLE III
Detailed Results of Study on Glycolysis in Human Blood

Experiment No.	Manometry,* c.mm. per hr. per ml.		Chemical determinations, γ per hr. per ml whole blood											
			Lactic acid						Glucose					
			Anaerobic			Aerobic			Anaerobic			Aerobic		
	Anaerobic	Aerobic	Initial	Final	Amount formed	Initial	Final	Amount formed	Initial	Final	Amount utilized	Initial	Final	Amount utilized
20	49.1	40.0	196	296	100	191	292	101	2160	2020	140	2220	2120	100
21	41.6	30.0												
22	40.3	35.6												
27	53.0	38.3	291	479	188	252	398	146						
28	45.2	30.0	212	398	186	175	305	130						
29	66.6	40.8	260	394	134	206	336	130	580	418	162	595	531	64
30	62.0	47.4	288	539	251	248	395	147						
31	53.4	28.1	284	430	146	282	421	139	445	310	135	500	378	122
32	70.5	53.2	216	424	208	212	349	137						
33	48.1	60.0	248	403	155	196	334	138	535	298	237	565	357	208
34	40.5	44.3	189	336	147	186	292	106	1760	1510	250	1850	1700	150
35	43.1	32.8	226	350	124	179	286	107						
36			177	341	164	135	280	145	1410	1210	200	1470	1330	140
36B			206	398	192	187	318	131						
37	44.5	45.9							1410	1210	200	1390	1290	100
38	47.1	40.8	190	344	154	187	276	89	1820	1650	170	1800	1720	80
39	41.7	62.3	306	439	133	247	368	121						
40	36.6	53.0	184	384	200	184	342	158	1520	1430	90	1610	1470	140
Average	48.9	42.6			165			128			176			123

* Values represent manometric readings corrected for retention.

TABLE IV
Summary of Eighteen Experiments

	Average	Range
$Q_{O_2}^{N_2}$	+0.28	+0.23 to +0.38
$Q_{O_2}^{Gluc}$	+0.25	+0.18 " +0.33
$Q_{H^+}^{N_2}$	+1.02*	+0.63 " +1.42
$Q_{H^+}^{Gluc}$	+0.81*	+0.54 " +0.98
$Q_{O_2}^{H^+}$	-1.08*	-0.56 " -1.44
$Q_{H^+}^{Gluc}$	-0.75*	-0.43 " -1.06

* These values are computed in terms of micrograms of lactic acid (H Lac) or glucose (Gluc) per mg. of cell protein per hour, with a positive sign indicating production and a negative sign indicating disappearance.

each experiment. The manometric readings have been corrected for the retention of CO_2 by the plasma. The chemical analyses considered unreliable, either because of large error in recoveries or poor agreement between duplicate determinations, have been omitted from Table III. To facilitate comparison between the various analytical methods used, the data of Table III are presented as a scatter graph in Fig. 2, with all manometric values expressed in terms of equivalent micrograms. Table IV summarizes the findings of the eighteen experiments reported in detail. Here the experimental results have been corrected on the basis of the cell

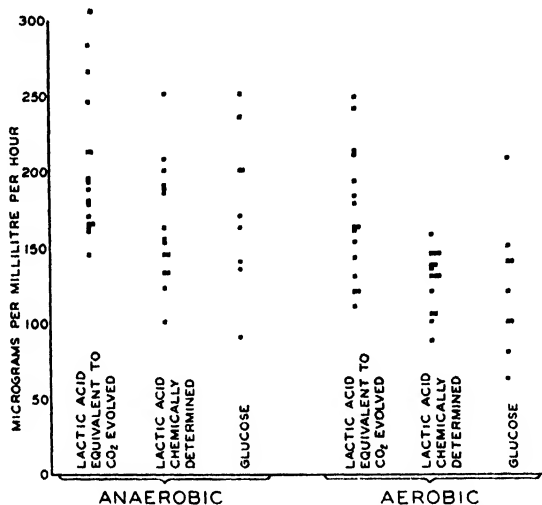


FIG. 2. Comparison of manometric and chemical data of eighteen experiments on anaerobic and aerobic glycolysis in human blood.

counts and are reported in terms of Q values of a hypothetical blood containing 5×10^6 red blood cells and 5×10^3 white blood cells per c.mm.

The following facts are apparent from these data. Anaerobic glycolysis exceeded aerobic in the large majority of the experiments. The evolution of CO_2 under anaerobic conditions was greater than under aerobic conditions in eleven of sixteen instances, and lactic acid formation was greater anaerobically in fourteen of fifteen experiments. In ten of thirteen experiments, the evolution of CO_2 was greater, both anaerobically and aerobically, than that which could be accounted for by lactic acid formation. The utilization of glucose was greater anaerobically in eight of nine determinations. More glucose disappeared than could be accounted for by the formation of lactic acid in six of eight experiments under anaerobic conditions, but this was a significant finding in only two of eight experiments when the

blood was treated aerobically. In the initial twenty experiments, not reported in detail, manometric data and chemical analyses conformed point by point to the above pattern.

DISCUSSION

The rate of glycolysis, both aerobic and anaerobic, as reported in this paper is of the same order of magnitude as has been reported previously. It can be defined no more precisely and is small in comparison with that of other tissues. The question arises as to whether this small glycolytic rate could not be accounted for entirely by the few white blood cells present in each ml. of whole blood. The density of human red cells is reported as 1.1 and their water content as 64 per cent (15). With these values, if one assumes that the total glycolysis is due to the erythrocyte, Q_G^N may be calculated to be +0.25, and Q_G^O +0.22. Such values are one-eighth less than the observed glycolytic rates reported in Table IV. Such close agreement would seem to indicate that the major part of the glycolysis measured in these experiments is the contribution of the red blood cell. Can the relative rôle of the white cell be approximated? Working with rabbit exudate leucocytes, Gilder¹ finds the average dry weight of 10^6 leucocytes to be 0.118 mg., and an average Q_G^N value of +24.4 and Q_G^O value of +15.9. Making two assumptions, *i.e.* that the rate of glycolysis for the rabbit leucocyte and for the human white cell is of the same order of magnitude, and that the exudate leucocyte resembles in its glycolysis similar cells circulating in the peripheral blood, one may apply Gilder's data to the results reported above. If the erythrocytes were inert and the total glycolysis were due to the white cells, the Q value for anaerobic glycolysis would be +48, for aerobic glycolysis +42. Since in 1 ml. of whole blood the total number of leucocytes represents approximately 10^{-3} of the total cell count, in whole blood the contribution of the leucocytes to the observed Q value might be said to approximate +0.04. Such a value added to the theoretical contribution of the red cell, *i.e.* +0.25 and +0.22, gives a value for the Q for whole blood very close to the Q determined experimentally, as reported here. Thus we feel that in whole human blood the relative contribution of erythrocyte and leucocyte to the total glycolysis is in the order of 8:1, while their numerical relationship is approximately 1000:1. Maclean and Weir (16) in 1915 stated that the glycolytic activity of the leucocyte was 200 to 1000 times as great as that of the erythrocyte.

That anaerobic glycolysis in whole blood is greater than aerobic glycolysis is evidenced by a greater evolution of CO_2 and by a greater formation of lactic acid. Such a conclusion based on manometric data alone might

¹ Gilder, H., personal communication.

be questioned, since aerobic glycolysis as measured in these experiments does not take into consideration the O_2 consumption and the CO_2 respiratory evolution of the cells present. Hence, manometric measurements made as described here are subject to a fundamental error which increases with increase in respiratory activity of the tissue present. In a tissue such as whole blood, however, with a leucocyte count not exceeding 15,000 cells per c.mm., respiration is admittedly small (17) and direct manometric measurement of aerobic glycolysis undoubtedly gives values which closely approximate the true values. This is evidenced by the close parallel between the analyses for lactic acid production and the manometric findings. That the CO_2 is uniformly evolved in excess of that which can be accounted for by the formation of lactic acid implies the formation of some other acid in addition to lactic acid.

From the initial experiments the impression was gained that more glucose was utilized than could be accounted for by lactate formation. Such also is the impression gained from previous reports in the literature. When the chemical data of the present study were scrutinized for their analytical accuracy by means of per cent error in recoveries of known additions, many glucose determinations had to be omitted. The remaining values showed a wide range of variation, but the spread corresponded to the distribution of the lactic acid analyses. Thus from the data presented glucose utilization would seem to equal, or nearly equal, lactic acid formation.

Grateful acknowledgment is made to Dr. W. H. Summerson and Dr. C. O. Warren for their interest and help in connection with the material presented here.

SUMMARY

1. Thirty-eight experiments on the anaerobic and aerobic glycolysis in whole human blood are reported. Manometric determinations are correlated with chemical analyses for glucose utilization and lactic acid formation.

2. Anaerobic glycolysis exceeds aerobic glycolysis, whether determined manometrically by the evolution of CO_2 or chemically by the formation of lactic acid.

3. The evolution of CO_2 exceeds the production of lactic acid, suggesting the formation of additional acids.

4. Glucose utilization roughly parallels lactic acid formation.

5. The relative contribution of erythrocytes and leucocytes to the total glycolysis is discussed.

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EFFECT OF CALCIUM ON THE INHIBITION OF THE SUCCINIC OXIDASE SYSTEM BY *d*- α -TOCOPHERYL PHOSPHATE*

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The involvement of vitamin E in muscle metabolism has been postulated on the basis of its action in nutritional muscular dystrophy. This condition is induced by an insufficient intake of vitamin E and can be cured, except in its terminal stages, by the administration of vitamin E. Particular attention has been paid to α -tocopheryl phosphate (α -TPh) because of its water solubility. This investigation was undertaken in an attempt to rationalize conflicting reports concerning the *in vitro* addition of α -TPh on the succinic oxidase system and as an attempt to ascertain the mechanism by which it exerts its effect.

In this contribution a mechanism is developed for the action *in vitro* of *d*- α -TPh on the succinic oxidase system, based on the ability of α -TPh to combine with calcium ions. Inhibition of the succinic oxidase system by α -TPh may be explained by the resulting low calcium level. Conflicting data obtained by previous investigators are rationalized and the relationships of calcium and vitamin E to muscle dystrophy are discussed.

Several investigators have related α -TPh and the enzymatic oxidation of succinic acid. Houchin (1) indicated that high succinic dehydrogenase values found in muscle tissue from dystrophic hamsters were lowered by *in vitro* addition of α -TPh. Govier, Bergmann, and Beyer (2) showed that α -TPh inhibits the succinoxidase system obtained from the skeletal muscle of normal animals. More recently, Govier, Yanz, and Grellis (3), in relating vitamin E and congestive heart failure, postulated that α -TPh inhibited DPNase (diphosphopyridinenucleotidase, coenzyme I nucleotidase). Houchin (4) reported uniform inhibition of α -TPh on succinoxidase, cytochrome oxidase, and cytochrome reductase systems. Basinski and Hummel (5) did not confirm the earlier work of Houchin (1), and reported that the succinic dehydrogenase activity of dystrophic hamster muscle homogenate is no different from that in normal tissue. Morgulis and Jacobi (6) have recently made the hypothesis that the calcium salt of α -TPh was involved in the *in vitro* effect of α -TPh on dystrophic muscle by modifying the adenosinetriphosphatase activity.

* Communication No. 114 from the Laboratories of Distillation Products, Inc., Rochester, New York.

The necessity of adding calcium ion for optimum activity in the aerobic determination of succinic dehydrogenase in tissue homogenates was discovered by Axelrod, Swingle, and Elvehjem (7). They further showed (8) that the "calcium effect" was indirect and depended on the acceleration of the destruction of DPN (diphosphopyridine nucleotide, coenzyme I, cozymase), probably by activating DPNase. Subsequent investigators have found this to be a satisfactory hypothesis; for instance, the extent of inhibition of succinic oxidase by cystine (9) is dependent on the incubation of tissue homogenate because of the destruction of DPN, which results in a uniform concentration of contaminating 4-carbon dicarboxylic acids.

Methods

Normal male albino rats, approximately 8 weeks old, weighing 200 gm. and maintained on stock ration were used. Animals were stunned by a blow on the head, decapitated, and drained of blood for a short time. The tissues were rapidly removed, rinsed with glass-redistilled water, and immediately packed in finely cracked ice. After cooling, bits of fat and connective tissue were removed, the tissues were blotted between moistened filter papers, and portions were rapidly weighed on a delicate torsion balance. A homogenate of the tissue was prepared in ice-cold glass-redistilled water by means of the device described by Potter and Elvehjem (10) with a pre-chilled tube and pestle. The homogenate was immediately pipetted into the reaction vessels to which all other reactants had been previously added. Dry weights of tissue were determined by evaporation to constant weight in open crucibles in an electric oven at 110°.

A conventional Warburg constant volume respirometer at 37.0° was used in all experimental and analytical work and the pH was determined with a Beckman pH meter (glass electrode). The activity of the succinic oxidase system was determined by the method of Schneider and Potter (11) in which the components of the final reaction mixture (pH 7.4) are as follows: 0.40 ml. of 0.25 M sodium phosphate buffer (pH 7.4); 0.20 ml. of 2×10^{-4} M cytochrome *c*; 0.30 ml. of 0.50 M sodium succinate; 0.10 ml. of 0.0120 M calcium chloride; 0.10 ml. of 0.0120 M aluminum chloride; 0.40 ml. of a 2.0 per cent homogenate of rat liver; and glass-redistilled water to make a total volume of 3.0 ml. The gas phase was air; 0.20 ml. of 10 per cent potassium hydroxide and a small strip of filter paper were placed in the center well to absorb carbon dioxide. The consumption of oxygen was measured manometrically. The solution of sodium succinate was placed in the side arm and added after equilibration. Additions of other components were made at the expense of water. Commercially prepared c.p. compounds were used throughout without further purification with the following exceptions: Cytochrome *c* was prepared in these laboratories

from beef heart by a modification¹ of the method of Keilin and Hartree (13) and dialyzed against glass-redistilled water. The sodium salt of *d*- α -TPh had been prepared in these laboratories.²

EXPERIMENTAL

The calcium salt of α -TPh was previously reported by Eppstein and Morgulis (14) to be insoluble, but it was necessary to determine whether conditions present in the enzymatic analysis would result in combination between α -TPh and calcium ion. Varying amounts of calcium chloride were added to a series of flasks containing a uniform concentration of α -TPh.

TABLE I
Spectrophotometric Determination of α -Tocopheryl Phosphate in Presence of Calcium Chloride

The *d*- α -tocopheryl phosphate concentration is 2.6×10^{-4} M throughout.

CaCl ₂ concentration $\times 10^4$	Ratio, <i>d</i> - α -TPh:CaCl ₂	d^* per cent of control	σ^\dagger
M			
0	1:0	100	
0.52	1:0.2	116	6.5
1.3	1:0.5	168	2.0
2.6	1:1	133‡	4.0
5.2	1:2	1§	
13.0	1:5	0§	
26.0	1:10	0§	

* Optical densities were determined at 286 $m\mu$ in a 1 cm. cell with a Beckman ultraviolet spectrophotometer.

† $\sqrt{\sum d^2/(N-1)}$.

‡ The filtrate was turbid and definitely colloidal in appearance.

§ Flocculent precipitate was formed

At molar ratios of calcium chloride to α -TPh of 2 or more to 1, a white flocculent precipitate formed. The mixtures were filtered through Whatman No. 40 paper and the filtrates analyzed spectrophotometrically for α -TPh. The data presented in Table I show that in aqueous solution, when the calcium concentration was 2 or more times greater than the α -TPh level, complete removal of α -TPh was effected. At lower concentrations

¹ The method is essentially that given by Potter (12), except that in the first and second filtrations a Sharples supercentrifuge was used.

² Appreciation is expressed to Dr. J. G. Baxter for a generous supply of sodium *d*- α -tocopheryl phosphate. The preparation had an $E_{1\text{cm}}^{1\%}$ at 286 $m\mu$ of 34.9 and was estimated to be 90 per cent pure.

of calcium chloride, an unexplained anomaly was observed, characterized by optical densities higher than the control values.

After the precipitates had been thoroughly washed and dried, they were decomposed with hot concentrated nitric acid. The bulk of the acid was then evaporated and the residue taken up in redistilled water. Elementary analysis showed the precipitate to be the monocalcium salt of α -tocopheryl phosphate.

$C_{15}H_{41}O_5PCa$. Calculated, Ca 7.30, P 5.65; found, Ca 7.34, P 5.33

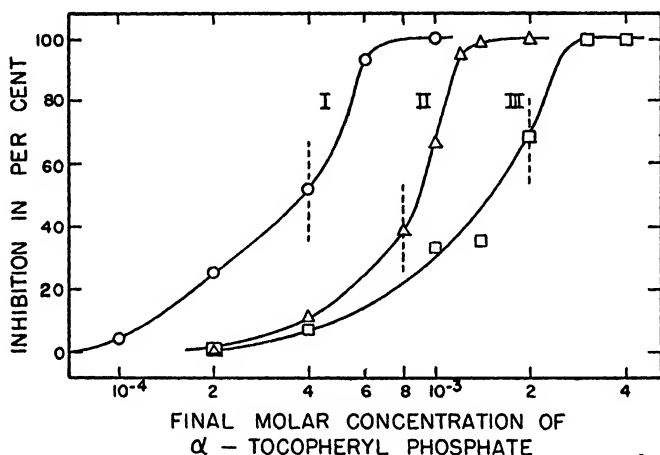


FIG. 1. Effect of calcium on the α -tocopheryl phosphate inhibition of the succinic oxidase system. Curve I, (O), $CaCl_2$ concentration of 4×10^{-4} M; Curve II, (Δ), $CaCl_2$ concentration of 8×10^{-4} M; Curve III, (\square), $CaCl_2$ concentration of 20×10^{-4} M. The intersection of the vertical dotted line and the curves indicates that the solution is equimolar in $CaCl_2$ and α -TPh. Calcium was added after α -TPh, which was added at the expense of water. Other components of the succinic oxidase assay are given in the text.

Since α -TPh reacts with calcium ions and, if present in sufficient concentration, would remove them from solution, determinations were made to see whether the α -TPh inhibition of the succinic oxidase system could be relieved by the addition of more calcium chloride. Series of determinations were made at varying levels of α -TPh and at three levels of calcium chloride, as diagrammed in Fig. 1. The higher the concentration of calcium chloride, the higher the α -TPh concentration must be to inhibit the system to the same degree. The inflection points of the inhibition curves (about 50 per cent inhibition) occur at approximately the point of 1:1 M ratio of α -TPh to calcium chloride, which agrees with the known structure of the calcium salt. It is evident that sufficient calcium chloride can com-

pletely relieve the inhibition of the succinic oxidase system occasioned by the addition of α -TPh.

TABLE II
Effect of Sequence of Calcium Addition on Inhibition Reaction

d - α -TPh concentration $\times 10^4$	CaCl ₂ concentration $\times 10^4$	Per cent inhibition	
		CaCl ₂ added before d - α -TPh	CaCl ₂ added after d - α -TPh
<i>M</i>	<i>M</i>		
4.0	4.0	85	52
4.0	8.0	52	12
4.0	20.0	56	6
4.0	40.0	44	0
10.0	8.0	88	68
10.0	20.0	84	27

The order of adding reactants is as follows: phosphate buffer, cytochrome *c*, aluminum chloride, calcium chloride (if added before α -TPh), succinate, α -TPh, calcium chloride (if added after α -TPh), water, and homogenate. Other details of the succinic oxidase assay are given in the text.

TABLE III
Effect of Glutamic Acid and DPN on α -Tocopheryl Phosphate Inhibition of Succinic Oxidase System

Experiment No.	d - α -TPh concentration $\times 10^4$	CaCl ₂ concentration $\times 10^4$	Glutamic acid concentration	DPN* concentration <i>mg. per 3 ml.</i>	Per cent inhibition
	<i>M</i>	<i>M</i>	<i>M</i>		
1	4.0	4.0			54
2	4.0	4.0	(L) 0.0067		37
3	4.0	4.0	(D) 0.0067		54
4	6.0	4.0			93
5	6.0	4.0	(L) 0.05†		67
6	4.0	8.0			12
7	4.0	8.0		1.0	54
8	4.0	8.0		1.0†	63

Components of the succinic oxidase assay are given in the text. α -TPh, glutamic acid, and DPN solutions were added at the expense of water.

* Schwarz DPN.

† Added 20 minutes after succinate.

The addition of any substance that will combine with calcium should function in the same way as α -TPh. This is illustrated in Table II, showing the effect of the sequence of the calcium addition on the extent of inhibition. Phosphate in the buffer reacts with calcium ion when it is added before α -TPh, particularly at the higher concentrations of calcium

chloride. This results in a lower level of calcium which is then more completely removed on addition of α -TPh. The observed inhibitions are higher than normal and are almost independent of the amount of calcium used. The inhibition of the succinic oxidase system was much less when α -TPh was added before calcium chloride.

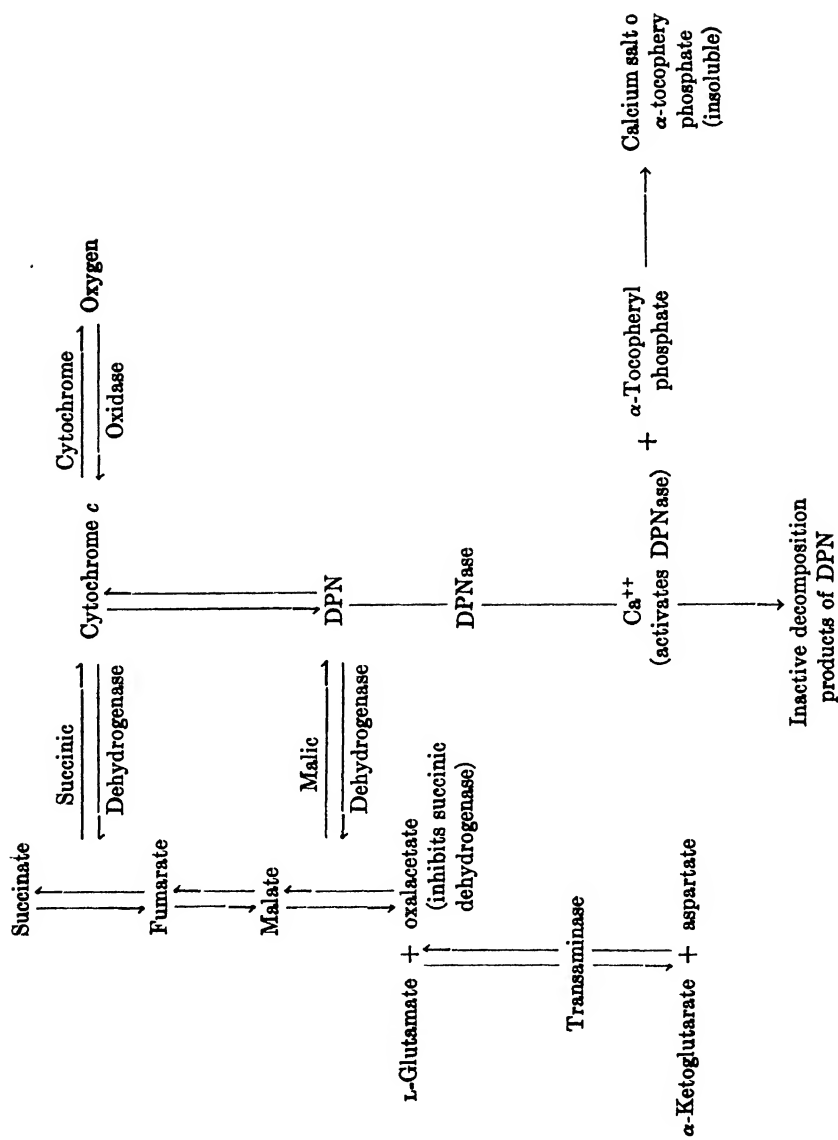
The previously reported stimulation by L-glutamic acid and the inhibition by DPN of the succinic oxidase determination were studied with an α -TPh-inhibited system (Table III). The addition of L-glutamic acid to a strongly inhibited succinic oxidase system partially relieved the inhibition, but the addition of D-glutamic acid was without effect. The addition of DPN to a succinic oxidase system slightly inhibited by α -TPh resulted in a pronounced increase in the extent of inhibition. The addition of DPN after the reaction had proceeded for 20 minutes resulted in a greater increase in the inhibition, probably because its specific substrate was in greater concentration and the DPN could function more effectively before it was destroyed.

DISCUSSION

The effect of calcium on the succinic oxidase system may be indicated by the accompanying diagram.

As a result of this investigation it would appear that α -TPh may be inhibiting the succinic oxidase system indirectly by reducing the calcium concentration and permitting the formation of oxalacetate. This hypothesis is substantiated by the fact that α -TPh unites with calcium ions in aqueous solution at levels comparable with those in the succinic dehydrogenase assay. Addition of calcium relieves the inhibition occasioned by the presence of α -TPh. The extent of inhibition varies inversely with the concentration of added calcium chloride at any one α -TPh level. This is further illustrated by the effect of the sequence in which calcium and α -TPh are added on the extent of inhibition. When calcium is added prior to α -TPh, any amount above a certain critical level is precipitated as the phosphate. As a result a greater inhibition is observed than would be obtained when the same amount of calcium is added after α -TPh.

According to the accompanying diagram, L-glutamic acid would be expected to relieve any inhibition occasioned by the formation of oxalacetate and DPN would be expected to increase the inhibition. Active transaminase necessary for this reaction is known (15) to be present in homogenates of normal rat liver. Oxalacetate would transaminate with L-glutamate, and, as oxalacetate was removed, inhibition of succinic dehydrogenase would decrease. Since at equilibrium this reaction shows 75 per cent transamination (16), an appreciable reduction in inhibition would be observed. The data show that the addition of L-glutamic acid



results in decreased inhibition but D-glutamic acid, which does not function in the transaminase system, has no effect (Table III). On the other hand, the addition of DPN should result in increased inhibition, since more oxalacetate would be formed if the malate system were functioning. The data show a marked increase in inhibition upon the addition of DPN, and a greater effect when the addition is delayed until the malate concentration increases and is available for reaction before the added DPN is destroyed by the previously activated DPNase.

The following mechanism is proposed to explain in part the inhibition of the succinic oxidase system by α -TPh. When α -TPh is added *in vitro*, it combines with calcium, which is an integral part of the succinic dehydrogenase assay method of Schneider and Potter (11). The calcium concentration is decreased below the critical level necessary to activate DPNase, and the DPN-linked malate system functions as a result. Oxalacetate produced in the malate system inhibits succinic dehydrogenase, with the over-all effect of the inhibition of the succinic oxidase system on the addition of α -TPh. This mechanism does not preclude the possibility of α -TPh directly inhibiting the succinic oxidase system, but a considerable portion of the observed inhibition can be attributed to its indirect action on the calcium concentration. Any assay method with calcium, magnesium, or other alkaline earth metal as part of the reaction mixture is not suitable for the determination of the effect of the addition of α -TPh.

The conflicting data of Houchin (1) and Basinski and Hummel (5) may be rationalized on the basis of the above mechanism for α -TPh inhibition of the succinic oxidase system. Houchin determined succinic dehydrogenase by an early method and used 20 γ per cent³ of calcium in the buffer (or a maximum concentration of 0.6 γ of calcium per flask). Basinski and Hummel, on the other hand, using the same method as the author, employed the addition of calcium chloride to a final concentration of 4×10^{-4} M (48 γ of calcium per flask).³ Houchin's succinic dehydrogenase determinations of normal tissues were low, since sufficient calcium was not present, but when tissues from dystrophic animals were used, containing considerable calcium, sufficient calcium was present in the homogenate itself to raise the assay values to the true levels. As a result, the inhibition produced by α -TPh was interpreted as restoring the activity to a normal which in itself was low. Their conflicting data, therefore, can be explained entirely on the basis of the difference in the calcium level employed.

Govier *et al.* (3) found that the addition of α -TPh to a vitamin E-deficient lactic dehydrogenase system resulted in a marked inhibition and apparently also protected against breakdown of DPN. These and similar ob-

³ Basinski and Hummel (5) erroneously reported Houchin's calcium concentration as 20 mg. per cent and Schneider and Potter's as 20 mg. per flask.

servations were interpreted as resulting from an inhibition of DPNase. On the basis of the present data, it would seem that this inhibition may be explained by the lack of activation of DPNase, due to low calcium levels.

In addition to his observations on tissue homogenates, Houchin (1) reported experiments in which the high oxygen consumption of dystrophic rabbit and hamster muscle (17) was lowered 40 per cent and toward normal by the addition of α -TPh to the medium. A high calcium level would result in an increase in activity of enzyme systems needing calcium for activation, *e.g.* DPNase, accompanied by an inhibition of DPN-linked systems, *e.g.* malic dehydrogenase. In view of the previously reported high calcium content of dystrophic muscle (18), the marked effect of calcium in modifying enzymatic activities (6, 7), and the ability of α -TPh to combine with and remove calcium, the abnormally high oxygen uptakes observed in dystrophic muscle slices may be attributed to the increased calcium deposition in the dystrophic animal.

SUMMARY

1. A reaction occurs in aqueous solution between calcium chloride and *d*- α -tocopheryl phosphate at a molar ratio of 2 or more to 1, which completely removes *d*- α -tocopheryl phosphate from solution by the formation of the monocalcium salt.

2. Inhibition of the succinic oxidase system by *d*- α -tocopheryl phosphate can be relieved by adding calcium chloride. The extent of inhibition varies inversely with the concentration of added calcium chloride.

3. When *d*- α -tocopheryl phosphate is added before calcium chloride, the inhibition of the succinic oxidase system is much less than when it is added after calcium chloride.

4. The addition of L-glutamic acid to a succinic oxidase system strongly inhibited by *d*- α -tocopheryl phosphate partially relieves the inhibition, but the addition of D-glutamic acid is without effect.

5. The addition of diphosphopyridine nucleotide to a succinic oxidase system slightly inhibited by *d*- α -tocopheryl phosphate results in a pronounced increase in the extent of inhibition.

6. A mechanism of *d*- α -tocopheryl phosphate inhibition of the succinic oxidase system is proposed, based on the removal of calcium ion and the resulting lack of activation of diphosphopyridinenucleotidase. The presence of diphosphopyridine nucleotide results in the inhibition of succinic dehydrogenase by oxalacetate formed from the diphosphopyridine nucleotide-linked malate system.

7. Conflicting results of previous investigators are rationalized and explained on the basis of the proposed mechanism.

8. The high oxygen uptakes observed in dystrophic muscle slices may be attributed to the increased calcium deposition in the dystrophic animal.

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TOCOPHEROLS (VITAMIN E) IN MILK: THEIR CHEMICAL DETERMINATION AND OCCURRENCE IN HUMAN MILK*

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The method of Quaife and Harris (1, 2) for the estimation of vitamin E in blood plasma has been adapted to the determination of total tocopherols in milk.

The quantities of milk, ethanol, and purified Skellysolve B (SS_B) used in the extraction mixture are increased proportionately as needed. For cow's milk the increase is 6-fold. An aliquot of the SS_B layer (*e.g.*, five-sixths of the total) is evaporated to dryness under nitrogen and the residue dissolved to a total volume of 10 ml. with a 1:1 mixture of ethanol-cyclohexane. This is hydrogenated, as previously described for blood plasma (2), for 1 to 3 minutes as needed to saturate the carotenoids with hydrogen, so that they do not interfere in the colorimetric assay for tocopherols. The tube is corked and centrifuged to separate the catalyst. 8 ml. of the supernatant are assayed by the Emmerie and Engel reaction, according to the technique previously described (1). 8 ml. of the same solvent are used for a blank. The vitamin E content is calculated from calibration data obtained with a solution of pure, natural *d*- α -tocopherol dissolved in 1:1 ethanol and cyclohexane.

Satisfactory recoveries (95 per cent) were obtained on adding α -tocopherol to the extraction mixture and assaying. This recovery, despite the large amount of fat contained in the Emmerie and Engel reaction solution, indicates little or no inhibition of the colorimetric reaction. This was confirmed by direct comparison of the colorimetric reaction mixture containing α -tocopherol with and without comparable amounts of butter fat. No color inhibition was found. Tocopherol cannot be concentrated from butter fat according to the molecular distillation technique as from other food fats (3) because of the large proportion of low molecular weight triglycerides which distil with it.

Values for a number of samples of winter cow's milk were determined by Swanson,¹ who found a range of 0.08 to 0.15 mg. of tocopherol per 100 ml. This corresponded to 17 to 30 γ per gm. of butter fat. A group of summer milks (4) had a mean of 42 γ per gm. of butter fat (about 0.17

* Communication No. 112 from the Laboratories of Distillation Products, Inc., Rochester, New York.

¹ Swanson, W. J., unpublished data.

mg. per 100 ml.). These values are in agreement with others in the literature which range from 25 to 44 γ per gm. of butter fat (5-7).

Fifteen samples of human milk, which were collected within 1 week after parturition, were assayed for vitamin E.² The values found range from 0.13 to 3.6 mg. per 100 ml. of milk or 76 to 1800 γ per gm. of fat. Twelve of the fifteen levels exceeded 200 γ per gm. of fat. Four composite samples of later human milk (from fourteen mothers in the 1st to 8th months of lactation) showed levels of 0.11 to 0.15 mg. of tocopherols per 100 ml. of milk, or 37 to 58 γ per gm. of fat. Thus the majority of the early milk samples assayed had much higher vitamin E levels than those of later samples of either human or cow's milk. Kofler has reported vitamin E levels in human milk on three samples, with the time of collection unspecified, to be 0.5, 1.6, and 3.6 mg. per cent (6).

SUMMARY

A simple method for the determination of vitamin E in milk is given which is a modification of the plasma method of Quaife and Harris (1, 2). Human milk samples which were obtained during the 1st week after parturition showed values of 0.13 to 3.6 mg. per 100 ml., the majority being much richer in vitamin E than the later milk samples, which had a mean level of 0.14 mg. per 100 ml.

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² The samples were obtained through the courtesy of Dr. W. J. Darby of Vanderbilt University School of Medicine, Nashville, Tennessee.

THE AMINO ACID COMPOSITION OF ANTERIOR HYPOPHYSEAL GROWTH HORMONE*

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(Received for publication, May 9, 1947)

A growth hormone, isolated from the anterior lobes of ox pituitaries by Li *et al.* (2, 3), was shown to be homogeneous by electrophoretic, diffusion, and solubility measurements. The molecular weight of this purified protein was estimated by an osmotic pressure method and the amino acid composition was determined by microbiological (glutamic acid) and colorimetric (cysteine, tyrosine, and tryptophan) procedures. More recently, Li (4) determined the cystine and methionine content of the growth hormone by colorimetric methods. In the present studies, the percentages of thirteen amino acids in this product have been determined by microbiological assay procedures.

EXPERIMENTAL

The purified preparations of the growth hormone were those described in Sections 4 (Sample L1-445E), 5 (Sample L1-498B), and 6 (Sample L1-464D) given in a previous paper (3). All weighings were made with the aid of a semimicro balance. Moisture was determined by heating approximately 12 mg. samples to constant weight at 70° in a vacuum oven. Acid hydrolysates for the determination of eleven amino acids (see Table I) were prepared by heating 38 to 80 mg. of sample and 2 ml. of 3.8 N hydrochloric acid in a sealed tube for 6 hours at 120°. Alkaline hydrolysates for the determination of tryptophan and tyrosine were prepared by heating 11 to 25 mg. of sample and 2 ml. of 5 N sodium hydroxide in a sealed tube for 10 hours at 120°. The microbiological assay procedures described previously were employed for the determination of arginine and tyrosine with *Lactobacillus casei* ϵ ; aspartic acid, glycine, lysine, and phenylalanine

* Paper 40. For Paper 39, see Rockland and Dunn (1). This work was aided by grants to one of us (M. S. D.) from the Nutrition Foundation, Inc., Difco Laboratories, Inc., Merck and Company, Inc., Standard Brands Incorporated, and the University of California (Los Angeles), and to one of us (C. H. L.) from the Rockefeller Foundation and the Research Board of the University of California (Berkeley). The authors are indebted to H. Block, M. N. Camien, and S. Shankman for technical assistance.

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with *Leuconostoc mesenteroides* P-60; glutamic acid, isoleucine, leucine, tryptophan, and valine with *Lactobacillus arabinosus* 17-5; and histidine and methionine with *Lactobacillus fermenti* 36. In all cases the assays were run in triplicate at five levels of sample with 3 ml. final volumes of solutions.

TABLE I
Per Cent of Thirteen Amino Acids in Three Preparations of Growth Hormone

Amino acid	Sample No.*		
	L1-445E†	L1-498B‡	L1-464D§
Arginine.	8.1	7.8	9.1
Aspartic acid	9.7	8.8	9.0
Glutamic "	12.9	12.6	13.0
Glycine	4.3	3.7	3.8
Histidine	2.6	2.6	2.65
Isoleucine . . .	3.8	3.3	4.0
Leucine	10.1	10.9	12.1
Lysine	6.9	6.7	7.1
Methionine	2.6	2.5	2.9
Phenylalanine	7.1	7.3	7.9
Tryptophan	0.97	0.83	0.84
Tyrosine	4.6	4.4	5.2
Valine.	4.5	3.9	3.9
Total	78.2	75.3	81.5

*Averages of six determinations found previously (3) with two growth hormone preparations: Colorimetric analysis, cysteine, 0.00 per cent, tryptophan, 0.92 per cent; tyrosine, 4.30 per cent; microbiological assay, glutamic acid 13.40 per cent. Averages of nine determinations found previously (4) by colorimetric analysis of four samples of growth hormone preparations: cystine 2.25 ± 0.05 per cent and methionine 3.06 ± 0.08 per cent. Within the limits of experimental error, methionine and cystine accounted for total sulfur (1.30 per cent) determined by the Carius method.

† Average of three values corrected for moisture (3.5 per cent) and ash (0.0 per cent).

‡ Average of two values corrected for moisture (6.6 per cent) and ash (0.0 per cent).

§ Single determination of phenylalanine, tryptophan, and tyrosine; each other figure, average of two values. All values corrected for moisture (5.2 per cent) and ash (0.0 per cent).

|| Alkaline hydrolysate; all others, acid hydrolysates

The response of the organisms was measured by titration with approximately 0.1 N alkali with bromothymol blue indicator.

The experimental and calculated results are given in Tables I and II. The probable relatively high reliability of the microbiological assay data is indicated by the mean deviations from the mean values at the different

levels of samples which ranged from 1.5 per cent for isoleucine to 7 per cent for tyrosine and averaged 3.8 per cent for the thirteen amino acids determined in Sample L1-464D. The average mean deviation from the mean

TABLE II

Molecular Weight, Amino Acid Residues Per Mole, and Corrected Per Cent of Amino Acids in Growth Hormone

Amino acid		Growth hormone (Sample L1-464D)						
Name	In growth hormone (Sample L1-464D)	Minimum mol. wt.*	Amino acid residues per mole				Minimum mol. wt. ‡	Amino acid in growth hormone
			Calculated†	Estimated	Calculated‡	Estimated		
	<i>per cent</i>							<i>per cent</i>
Arginine	9.1	1,914			24.4	24	1,950	8.93
Aspartic acid	9.0	1,479			31.7	32	1,462	9.10
Glutamic "	13.0	1,131			41.4	42	1,114	13.2
	13.4¶	1,098			42.7			
Glycine	3.8	1,977			23.7	24	1,950	3.85
Histidine	2.65	5,853	7.5	8	8	8	5,850	2.65
Isoleucine	4.0	3,275			14.3	14	3,343	3.92
Leucine	12.1	1,083			43.3	43	1,088	12.0
Lysine	7.1	2,059			22.7	23	2,036	7.18
Methionine	2.9	5,145	8.6	9	9.1	9	5,200	2.87
	3.1**	4,813	9.2		9.6			
Phenylalanine	7.9	2,091			22.4	22	2,127	7.77
Tryptophan	0.84	24,309	1.82	2	1.92	2	23,400	0.87
	0.92¶	21,750	2.03		2.15			
Tyrosine	5.2	3,845			13.5	12	3,900	4.64
	4.3¶	4,214			11.1			
Valine	3.9	3,003			15.6	16	2,925	4.00

* Mol. wt. of amino acid to per cent of amino acid in growth hormone $\times 100$.

† (44,250, mol. wt. found by Li *et al.* (3))/(minimum mol. wt.).

‡ 46,800 (estimated mol. wt.)/(minimum mol. wt.). Mol. wt. estimated from histidine data (5853 \times 8).

§ 46,800 to estimated amino acid residues per mole.

|| (Mol. wt. of amino acid $\times 100$)/(estimated minimum mol. wt.).

¶ Value found by Li *et al.* (3).

** Value found by Li (4).

was 3.5 per cent for these amino acids determined in Samples L1-445E and L1-498B.

DISCUSSION

The amino acid composition of the three products prepared by successive sodium chloride fractionation, pH and ammonium sulfate fractionation, and isoelectric precipitation is shown in Table I. It is of interest that the

types and proportions of amino acids in the growth hormone did not differ significantly from those in other common proteins. That the percentage of arginine, leucine, and some other amino acids in the three preparations differed considerably was not unexpected in view of the observation of Li *et al.* (3) that the final product (Sample L1-464D, but not any intermediate fraction, was homogeneous, as determined by the criteria (electrophoresis, diffusion, solubility, and biological potency) employed.

Data on the molecular weight, amino acid residues per mole, and corrected percentages of thirteen amino acids in the growth hormone (Sample L1-464D) are given in Table II. The calculations were based on the assumptions that the molecular weight (44,250) found by Li *et al.* (3) is approximately correct and that the percentage of histidine given in Table II is the true value. The latter hypothesis is supported by the recent report of Dunn and Rockland (5) that histidine may be determined with high precision and accuracy by the microbiological procedure employed in the present experiments.

A mole of growth hormone contained a minimum of 7.5 histidine residues, according to calculations based on the percentage (2.65) of histidine found in the present experiments and the approximate molecular weight (44,250) which Li *et al.* obtained by an osmotic pressure method. If it is assumed that there are eight histidine residues per mole, the estimated molecular weight of the growth hormone protein would be 46,800 and the corrected amino acid residues per mole would range from two for tryptophan to 43 for leucine. The percentages of the amino acids calculated from the respective corrected minimum molecular weights of the growth hormone are given in Table II. These values correspond closely to those determined by colorimetric and microbiological methods. Disagreement between the values found by these procedures was marked only in the case of one amino acid (tyrosine). Since the racemization of tyrosine may be incomplete by the alkaline hydrolysis employed in the microbiological assay procedure, it seems probable that the value for tyrosine found colorimetrically may be more nearly correct than that determined microbiologically.

SUMMARY

The percentages of thirteen amino acids in an electrophoretically pure preparation of protein obtained from the anterior lobes of ox pituitaries have been determined by microbiological procedures. The molecular weight has been estimated to be 46,800 by calculations based on the approximate molecular weight (44,250), determined by an osmotic pressure method, and the percentage of histidine (2.65 per cent) found by a microbiological

procedure. The number of amino acid residues per mole of growth hormone and the corrected percentages of amino acids have been determined.

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ON THE PROTEOLYTIC ENZYMES OF ANIMAL TISSUES

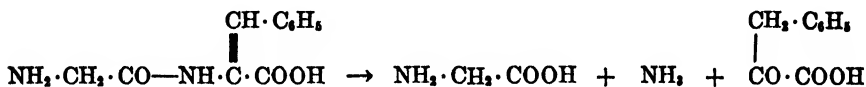
VI. DEHYDROPEPTIDASE*

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(Received for publication, May 1, 1947)

In 1932, Bergmann and Schleich (1) established the presence, in glycerol extracts of swine and sheep kidney and of commercial pancreatin, of an enzyme which they named dehydropeptidase. This enzyme was found to catalyze the hydrolysis of dipeptides of α, β -unsaturated amino acids. Thus, glycyldehydrophenylalanine (GdP) gave, on enzymatic hydrolysis, phenylpyruvic acid, ammonia, and glycine, according to the following equation:



Similarly, glycyldehydroalanine (GdA) was split to pyruvic acid, ammonia, and glycine. More recently, Greenstein and Leuthardt (2) have shown dehydropeptidase activity to be present in aqueous extracts of many normal and neoplastic mammalian tissues. The wide-spread distribution of this enzyme has given support to the possibility that peptides of α, β -unsaturated amino acids represent active participants in intermediary protein metabolism (3). For this reason, the purification of dehydropeptidase and a closer investigation of its properties have seemed desirable. The present communication deals with the kinetics and activation behavior of the enzyme, from rat kidney, which hydrolyzes GdP.

Spectrophotometric Assay of Dehydropeptidase Activity—In the initial studies on dehydropeptidase, Bergmann and Schleich followed the rate of hydrolysis by titration of the liberated carboxyl groups, according to the method of Grassmann and Heyde (4). Greenstein and Leuthardt measured the extent of hydrolysis by determination of the ammonia produced. Recently, Carter and Greenstein (5) have followed the action of dehydropeptidase on GdA by means of a spectrophotometric method in which there is measured the rate of the disappearance of the characteristic ultra-violet absorption of GdA at 2400 Å.

* This study was aided by grants from the American Cancer Society (on recommendation of the Committee on Growth of the National Research Council) and the Rockefeller Foundation.

† National Institute of Health Junior Research Fellow.

The experiments to be described in this paper have also involved the use of a spectrophotometric assay for enzyme activity, in which GdP served as the substrate for dehydropeptidase. As has been noted previously (6), the derivatives of aromatic dehydroamino acids exhibit striking ultraviolet absorption spectra in the region 2700 to 3300 Å and are especially suitable for biochemical studies in the presence of tissue extracts which absorb strongly near 2400 Å, the region in which the derivatives of aliphatic dehydroamino acids show selective absorption. It is apparent from Fig. 1 that, at pH 8, the ultraviolet absorption spectrum of GdP

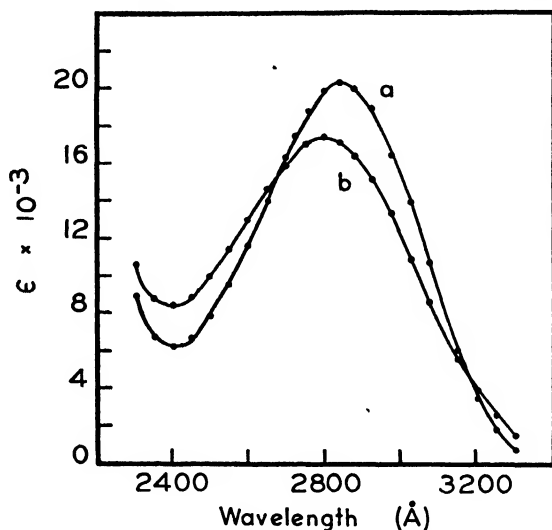


FIG. 1 Absorption spectra of glycyldehydrophenylalanine at various pH values. Curve a, pH 2; Curve b, pH 8.

has a high, sharp peak at 2800 Å. At this wave-length, the molecular extinction coefficient ϵ equals 17,440. At pH 2, GdP absorbs maximally at 2840 Å (ϵ equals 20,280). A mixture in equimolar proportions of the split-products of dehydropeptidase action, phenylpyruvic acid, glycine, and ammonia, shows, at pH 8, only slight absorption at 2800 Å (ϵ equals 458). Over a period of 1 to 3 days at 25°, phenylpyruvic acid may decompose slowly to form benzaldehyde, which, at pH 8, has an absorption coefficient of 1310 at 2800 Å. These data indicate the feasibility of a spectrophotometric assay of dehydropeptidase with GdP as the substrate.

Enzyme preparations were obtained from rat kidneys by grinding the tissue in a mortar with acid-washed sand. Water was then added (3.3 cc. per gm. of tissue ; the mixture was adjusted to pH 8 with *N* NaOH, and allowed to stand at room temperature for 1 hour with frequent stirring.

The extract was decanted, and to it there was added an equal volume of saturated sodium sulfate. The pH was then adjusted to 5 to 6 with N HCl, and the resulting precipitate was removed by filtration with the aid of Hyflo Super-Cel or by centrifugation. In this manner, there were obtained enzyme extracts of somewhat variable activity. When stored at 0°, the enzyme preparations retained their potency for several months. No preservative was used at any time.

TABLE I
Kinetics of Dehydropeptidase Action

Substrate, glycyldehydrophenylalanine (GdP) (5.7×10^{-5} M); pH 7.9 (0.05 M phosphate buffer); temperature, 25.3°.

Enzyme concentration	Time	E^*	ΔE^\dagger	Hydrolysis	$K_{\text{GdP}} \times 10^{\ddagger}$	$C_{\text{GdP}} \times 10^3$ (average)
<i>mg. protein N per cc.</i>	<i>min.</i>			<i>per cent</i>		
0.018	0	1.000				
	97	0.873	0.127	12.7	0.610	
	135	0.822	0.178	17.8	0.635	
	262	0.692	0.308	30.8	0.610	3.4
0.027	0	0.975				
	74	0.820	0.155	15.9	1.01	
	111	0.749	0.226	23.2	1.04	
	216	0.610	0.365	37.5	0.945	3.7
0.036	0	0.978				
	40	0.877	0.101	10.3	1.20	
	74	0.792	0.186	19.0	1.24	
	111	0.720	0.258	26.4	1.20	3.4

* Optical density ($\log I/I_0$) determined with the spectrophotometer. A control experiment without the substrate was used as the comparison blank.

† Decrease in optical density, $E_t - E_0$, where E_t is the optical density at time t and E_0 is the optical density at the start of the hydrolysis.

‡ $K = 1/t \log (100/100 - \text{per cent hydrolysis})$.

The test system for the assay of dehydropeptidase activity contained, per 5 cc. of reaction mixture, 2.5 cc. of 0.1 M phosphate buffer (pH 7.9), 1 cc. of a solution of the substrate (GdP·2H₂O (7)), and 0.05 to 0.2 cc. of the kidney extract. The substrate concentration in the test system is given in Tables I to IV. For each run, a comparison blank was set up as a control in which the substrate solution was omitted. Both the experimental and control flasks were incubated in a constant temperature water bath and removed at suitable time intervals for spectrophotometric measurement by means of a Beckman ultraviolet spectrophotometer. The rate of hydrolysis of GdP was determined from the optical density, at 2800 Å, of the mixture in the experimental flask, read against the cor-

responding comparison blank. The pH values of the incubation mixtures at the start and at the end of each run were checked by means of a glass electrode assembly.

Kinetics of Dehydropeptidase Action—Previous workers (1, 2) have followed the action of dehydropeptidase at 37–40°. It was noted in the present study that, at these temperatures, satisfactory rate constants could not be calculated from the hydrolytic data. When the experiments were conducted at a lower temperature (25.3°), however, the initial rate of enzymatic action accorded well with the kinetics of a first order reaction (cf. Table I). For this reason, all subsequent experiments on the rate of dehydropeptidase action were performed near 25°. From the data in Table I, it will be noted that the first order velocity constant K_{GdP} is proportional to the amount of enzyme in the test solution. The proteolytic coefficient ($'_{\text{GdP}}$ (defined as K_{GdP} per mg. of protein N per cc. of test solution (8)) may be used, therefore, to follow the purification of dehydropeptidase.

Examination of the kinetic data showed that, after about 35 per cent of the substrate had been hydrolyzed, the first order velocity constants decreased and, when 65 per cent hydrolysis had been attained, the rate was negligibly slow. It does not appear probable that this effect is due to the inhibitory action of the products liberated on hydrolysis since, when phenylpyruvic acid, glycine, and ammonia (concentration, 5×10^{-3} M) were added at the start of the reaction, no appreciable decrease in the initial rate of hydrolysis was observed. The possibility that the cessation of hydrolysis at 65 per cent might be due to the establishment of an equilibrium between GdP and its split-products is unlikely, since no increase in absorption at 2800 Å was observed when phenylpyruvic acid, glycine, and ammonia were incubated for 48 hours with dehydropeptidase at pH 7.9 and 25°. Similarly, no apparent synthesis of GdP could be noted when glycylamide was incubated with phenylpyruvic acid in the presence of dehydropeptidase. It has been postulated recently (9, 3) that keto acids may condense with amino acid amides in the presence of suitable tissue extracts. The possibility that the change in the kinetics of dehydropeptidase action upon prolonged incubation might be due to changes in the pH of the test solution was ruled out by determinations of the pH at the start and the end of the reaction.

pH Dependence of Dehydropeptidase Activity—Bergmann and Schleich (1) reported that the optimal pH for the action of glycerol extracts of pancreatin on GdP was near 7.8. In the present study, it was found that the pH optimum for rat kidney dehydropeptidase is at 8.0 (cf. Fig. 2). Of particular interest was the extremely sharp character of the pH dependence curve, which indicated that changes of 0.2 of a pH unit in the region pH 7 to 9 can markedly alter the rate of enzymatic action.

A study of the effect of pH on the stability of dehydropeptidase showed that it was rapidly inactivated at 25° at pH 3 or pH 11.5 (Table II), but

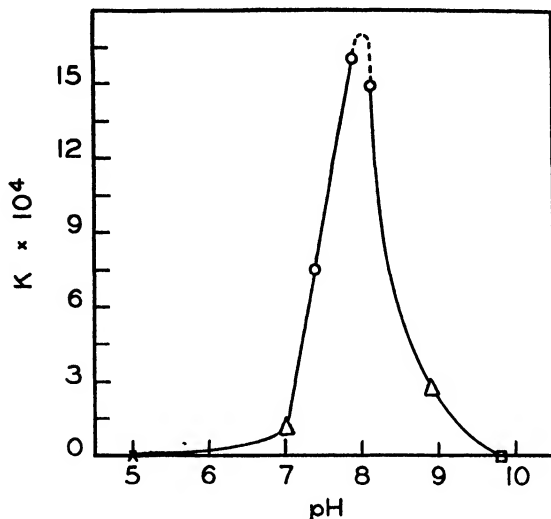


FIG. 2. pH optimum of dehydropeptidase. Concentration of buffers, 0.025 M. X, citrate buffer; O, phosphate buffer; Δ, phosphate-borate buffer; □, borate-carbonate buffer; temperature 25.6°. Enzyme concentration, 0.035 mg. of protein N per cc. of test system.

TABLE II
pH Stability of Dehydropeptidase at 25°

The pH of the enzyme solution (2 cc.) was adjusted by the addition of suitable amounts of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide and the volume was brought to 2.5 cc. After incubation for 2 and 25 hours at 25°, 0.2 cc. aliquots of each enzyme solution were removed and assayed for enzyme activity toward glycyldehydrophenylalanine (GdP) at pH 7.9, as described in Table I. Enzyme concentration, 0.034 mg. of protein N per cc. of test solution.

pH of enzyme solution	$K_{GdP} \times 10^3$	
	After 2 hrs	After 25 hrs.
3.2	0.000	
6.5	0.863	1.05
7.4	0.820	0.782
9.6	0.591	0.830
11.5	0.000	

retained its activity at pH values near neutrality. No satisfactory explanation can be offered at present for the slight increase in enzymatic activity noted following incubation for 25 hours at pH 6.5 or pH 9.6.

Effect of Phosphate on Dehydropeptidase Action—It has been reported by Carter and Greenstein (10) that the presence of graded concentrations of phosphate buffer of pH 7.1 results in an increasing inhibition of dehydropeptidase action on GdA, as the phosphate concentration is raised. Since the kinetic studies described in an earlier section of this paper involved the use of phosphate buffers, a reinvestigation of the reported inhibition by phosphate appeared necessary. It was found that kidney extracts which had been dialyzed against 3 per cent sodium chloride solution for 15 hours

TABLE III

Effect of Phosphate on Dehydropeptidase

To 1 cc. of glycyldehydrophenylalanine (GdP) solution (containing 0.072 mg. of GdP·2H₂O), there were added 0.2 cc. of kidney extract (containing 0.13 mg. of protein N) and enough water to make the volume 5 cc. The comparison blank, as in previous experiments, contained no substrate. At the start of the hydrolysis, and every 15 minutes thereafter, 0.2 cc. of 0.001 N sodium hydroxide was added to the experimental flasks, and 0.14 cc. of alkali plus 0.06 cc. of water was added to the blanks. It had been determined previously that these additions were required to maintain the pH of the unbuffered solutions at pH 7.9.

A second set of experiments was set up in which, in addition to substrate and enzyme solution, there were present 2.5 cc. of 0.1 M phosphate buffer (pH 7.9). At the same time that alkali was added to the unbuffered series, water (0.2 cc.) was added to the series containing phosphate. In this manner, the volumes of all the solutions were kept the same throughout the experiment.

The pH of each solution was determined electrometrically at the start and at the end of the experiment, and in all cases was found to be 7.8 to 7.9.

Time	Hydrolysis	
	No phosphate	Plus phosphate
min.	per cent	per cent
65	7.7	6.1
68	10.6	9.6
118	15.4	12.8
131	17.7	18.2

at 0° showed little difference in enzymatic activity toward GdP, whether phosphate or borate buffer (0.05 M, pH 7.9 to 8.0) was used in the test system. For the same kidney extract (0.2 cc. per 5 cc. of test solution), under the experimental conditions given in Table I, the K_{GdP} was found to be 6.1×10^{-4} in the presence of phosphate, and 6.4×10^{-4} in the presence of borate. To obviate the possibility that borate and phosphate have a comparable inhibitory effect, the hydrolytic reaction was also performed in unbuffered solution. In this case, however, it was essential to maintain the reaction mixture at pH 7.9 by the addition of 0.001 N sodium hydroxide during the course of the hydrolysis. Unless this is done, changes in the pH of the test solution, due to autolysis of the kidney extract, are of suf-

ficient magnitude to affect markedly the rate of enzymatic action (cf. Fig. 2). The data in Table III indicate that the initial rate of hydrolysis of GdP by rat kidney dehydropeptidase at pH 7.9 is essentially the same in the presence and the absence of phosphate.

The discrepancy between the data presented above and the conclusions drawn by Carter and Greenstein may well arise from the fact that these investigators conducted their studies in a system having an initial pH of 7.1 and containing no buffer other than the tissue extract, the substrate

TABLE IV
Inhibition of Dehydropeptidase

Substrate, glycyldehydrophenylalanine (GdP) (5×10^{-5} M); pH 7.9 (0.05 M phosphate buffer); temperature, 25.7°. Enzyme concentration, 0.046 mg. of protein N per cc. of test system. The comparison blanks (without substrate) contained the inhibitors in the same concentration as the comparable experimental flasks. The solutions of cysteine, cyanide, and sulfide were brought to pH 8 prior to their addition. The concentration of the neutralized sulfide solution was checked by iodometric titration.

Inhibitor	Concentration	$K_{\text{GdP}} \times 10^3$	Inhibition
	M		per cent
None		0.56	
Cyanide	0.010	0.00	100
	0.002	0.36	36
	0.001	0.54	4
Cysteine	0.010	0.12	79
	0.002	0.22	61
	0.001	0.41	27
Sulfide	0.0006	0.00	100
	0.0003	0.11	80

(GdA), or, as the hydrolysis proceeded, the split-products of enzymatic action. With the concentrations of substrate employed in their experiments (about 0.0125 M), it may be expected that, in the absence of added buffer, the ammonia and glycine liberated during the hydrolysis would tend to cause the solution to become more alkaline. In view of the marked dependence of the rate of dehydropeptidase action on pH, and the pH optimum near pH 8, such changes in alkalinity of the test solution would accelerate the reaction. The addition of increasing concentrations of phosphate buffer at pH 7.1 would tend to counteract this effect, and it is likely, therefore, that the observed rate of hydrolysis of the substrate would be slowest at the highest concentration of phosphate buffer.

Inhibition of Dehydropeptidase by Cyanide, Cysteine, and Sulfide—The data in Table IV confirm the report of Bergmann and Schleich (1) that cyanide is an inhibitor of dehydropeptidase action. It is of interest,

however, that, in order to attain approximately 50 per cent inhibition, the cyanide concentration must be as high as 0.002 M. It has not been possible, however, to confirm the report (1) that cysteine has no effect on dehydropeptidase. As will be noted from the data in Table IV, cysteine is approximately as effective an inhibitor of the enzyme as is cyanide. Sulfide, on the other hand, is a more powerful inhibitor of dehydropeptidase than is either cyanide or cysteine, since sulfide concentrations of 0.0006 M are sufficient to inhibit the enzyme activity completely. The pronounced inhibitory action of substances known to influence the enzymatic action of known metal-containing enzymes raises the possibility that dehydropeptidase belongs to this group of enzymes.

It has been postulated recently (2) that the hydrolysis of cystine peptides by liver extracts to form pyruvic acid, ammonia, and hydrogen sulfide from the cystine moiety of the peptide is due to the initial enzymatic desulfhydration of the cystine peptide to yield a peptide of dehydroalanine which then is cleaved by dehydropeptidase. The pronounced inhibition of dehydropeptidase by low concentrations of sulfide, reported in Table IV, suggests that this interpretation is subject to doubt.

Partial Purification of Dehydropeptidase—The further study of the properties of dehydropeptidase, and, in particular, its specificity of action, requires that an appreciable purification of the enzyme be effected. Although it has been assumed in this paper, and by previous investigators, that substrates such as GdP and GdA are split by the same enzyme, unequivocal evidence to prove this assumption is lacking. It has been reported (11) that, in addition to an enzyme which splits GdA, some animal tissues contain a dehydropeptidase which can hydrolyze chloroacetyldehydroalanine. It may be mentioned that Bergmann and Schleich (1) found the tripeptides glycyldehydrophenylalanylglycine and glycyldehydrophenylalanyl-L-glutamic acid (7) to be resistant to the enzymatic action of glycerol extracts of pancreatin. A similar result has been noted in the present study with extracts of rat kidney. It was found that the absorption of these two compounds at 2800 Å did not decrease markedly following incubation with dehydropeptidase for 26 hours at 25°.

In preparation for the closer study of the specificity of dehydropeptidase, experiments have been initiated on the purification of the enzyme. It has been found that a rat kidney extract which has a proteolytic coefficient C_{GdP} of 0.027, when dialyzed against distilled water for 21 hours at 0°, yields a copious precipitate. The extraction of this precipitate with 5 per cent sodium chloride gives a solution having a C_{GdP} of 0.14. Dialysis of the crude extract against saline instead of water gives no precipitation and results in no appreciable change in the proteolytic coefficient. It would appear, therefore, that dehydropeptidase activity is associated with the globulin fraction of the tissue extract.

SUMMARY

A spectrophotometric method was employed to determine the rate of hydrolysis of glycyldehydrophenylalanine by dehydropeptidase from rat kidney. The initial rate follows the kinetics of a first order reaction, the constant of which is proportional to the amount of enzyme. The activity of dehydropeptidase is sensitive to small changes in pH; the pH optimum is at pH 8. Cysteine, cyanide, and sulfide inhibit this enzyme.

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NOTE ON THE REACTION OF FORMALDEHYDE WITH COLLAGEN

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A series of papers on this subject was published in 1942-45 by Theis and his collaborators (1-5). Since the results of these investigations and their interpretation differ considerably from the findings of others (6-10), attention will be called to errors inherent in the technique employed by Theis. Further, the interpretation of the findings and the theoretical deductions do not agree with our present knowledge of the reactivity of collagen.

Theis applies the pressing technique (11, 12) to the formaldehyde-tanned collagen preparations in order to remove unbound formaldehyde. It was claimed by McLaughlin (11, 12), the originator of this method, that by pressing the treated protein twice at 5000 pounds pressure per sq. in. a complete removal of free water and electrolytes was effected. Theis, using twice the pressure mentioned, has evidently accepted this claim (3). However, direct experimental evidence for its justification has been presented neither by McLaughlin nor by Theis. Certain errors in the pressing method were pointed out by the present writer in 1938 (13), in regard to chrome tanning investigations, which were later fully substantiated by McLaughlin and Adams in 1942 (14). The "bound" water of the pressed stock, averaging 125 per cent water on the basis of protein, is composed of about 20 per cent *bound* water, not available as solvent (15), and about 100 per cent free water present in the form of the solution in contact with collagen. In stock in equilibrium with solutions of high content of solute, very large errors are introduced by analysis of the pressed stock, since the unbound tanning agent is included in the figure for the amount of agent irreversibly bound by protein.

In their review of the reaction of formaldehyde with proteins, French and Edsall (16), seeking an explanation of the disagreement of Theis' data with the findings of recent investigations (6-10) point to the possibility of the presence of considerable amounts of uncombined formaldehyde in pressed protein, previously exposed to formaldehyde solution. Their explanation is confirmed by the author's experiments. In the standard procedure of Theis, 2 gm. of protein are treated in 200 ml. of 1 per cent aqueous formaldehyde solution. Since the formaldehyde fixation is of the order of 1 to 2 per cent CH_2O on a collagen basis, the original formal-

dehyde concentration is not markedly changed by the reaction. The pressed stock, containing something like 1 ml. of 1 per cent CH_2O solution (= 1 per cent CH_2O) figured on 1 gm. of protein, is air-dried and analyzed. With a volatile substance, such as formaldehyde, part of the sorbed substance will evaporate during the drying, the extent depending upon the mode of drying and the physical condition of the hide substrate; that is, whether flaccid, swelled, or shrunk (denatured). The errors in the amount of bound formaldehyde will thus vary within limits, depending upon these secondary factors. This criticism of data obtained by the pressing technique also applies to investigations of the acid- and alkali-combining capacity of collagen, in the native and the denatured state, given by Theis (2).

In the interpretation of the curve of fixation of formaldehyde by collagen, given by Theis, some well established facts have been overlooked. It is claimed that, in the range pH 1 to 6.5, the bound formaldehyde will function as bridges between adjacent collagen chains by condensation with weakly basic imino groups in juxtaposition. This reaction is said to occur over the whole pH range and is considered to account mainly for the increased structural resistance of the collagen-formaldehyde compound. Fixation of formaldehyde, independent of the pH value of the system and probably involving peptide linkages, is an established fact. However, this type of reaction, mainly occurring in rather concentrated solutions of formaldehyde, does not stabilize the protein lattice. This is shown by the fact that collagen with its basic groups completely inactivated by polymethylene naphthalenedisulfonic acid, which is specific for the acid-binding protein groups and does not interfere with peptide linkages, is not stabilized with the formaldehyde taken up, which is probably attached to peptide groups (9, 10). Further, formaldehyde in combination with deaminized collagen does not improve the rigidity of the structure in the pH range <7 , although swelling of the structure is absent and the steric conditions of the protein backbone are favorable for such bridge formation (9, 10).

Evidence for the participation of amino groups from pH 4 to 7 has been adduced by Highberger and his coworkers, Bowes and Pleass, and the present author (6-10). It can safely be stated that ϵ -amino groups of lysine residues are an absolute requirement for the stabilization of collagen by formaldehyde. If the bridging further involves an adjacent peptide group, as Nitschmann and Hadorn (17) believe, that does not change this fact.

This hypothesis of the formation of methylene bridges between ϵ -amino groups of lysine and the nitrogen of adjacent peptide linkages removes the objection of steric nature to a methylene bridge between two amino groups on adjacent chains. It was pointed out in 1940 (9, 10) that the chance for two amino groups to approach each other closely enough for such a

reaction should be rather slight. Hence, it was suggested that only a minute part of the bound formaldehyde is bridge-forming, the main part reacting *without* interlocking of collagen chains, with the formation of $-\text{N}:\text{CH}_2$ and $-\text{NH}\cdot\text{CH}_2\text{OH}$ structures. The concept of Nitschmann and Hadorn does away with this objection, since the assumption of the proximity of amino and peptide groups on adjacent protein chains seems reasonable. Further, it also recognizes the bonding strength of the compound formed (16) and agrees quantitatively with the ratio of one CH_2O bound by one NH_2 group, present in formaldehyde-treated collagen in its zone of maximum stability (pH 6 to 8). The Nitschmann-Hadorn concept is by far the most satisfactory explanation of formaldehyde-protein systems yet advanced.

Further, experimental indications are at hand showing that, at low pH values, 1 to 3, formaldehyde reacts with collagen by discharge of the NH_3^+ groups ($-\text{NH}_3^+ \rightarrow -\text{NH}_2 + \text{H}^+$). High concentration of formaldehyde and extended time of interaction are necessary for such a reaction (9, 10). Fixation by acid amide groups, indicated to occur in certain proteins rich in amide N (18, 19), does not seem to enter into the reaction with collagen (limed hide) which contains only very small amounts of amide groups in this state (20, 21).

Between pH 7.0 and 9.5, Theis (2) believes reactions with the imidazole group of the histidine residue to be involved and further, at pH values greater than 9.5, the ϵ -amino group of lysine is assumed to bind formaldehyde. Even at the highest pH value covered, pH 12, reaction with the basic group of the arginine residue is said not to occur. Theis' explanation of the formaldehyde fixation in the range pH 7.5 to 9 cannot be correct in view of available data showing the influence of removal of amino groups by deamination on formaldehyde fixation. The interaction of lysine amino groups from pH 4 to 8 has been strikingly demonstrated in a number of investigations (6-10). Since collagen contains at the most only 0.6 per cent histidine (20, 21), formaldehyde binding by this group would account for only 0.04 mm of CH_2O per gm. of collagen, or less than one-tenth of the amount bound in the pH range concerned. However, it was shown by Gerngross in 1920 that the histidine group does not take part in the fixation of formaldehyde (22). Recent investigations show that formaldehyde, in reacting with histidine itself, is incorporated into the molecule, forming a closed ring which is resistant to sulfuric acid of the concentration used in the determination of bound formaldehyde (23).

Moreover, in allocating regions of such high pH values to the reactivity of lysine and arginine groups with formaldehyde as in Theis' hypothesis, due consideration has not been given to the possibility that the pK values of the ϵ -amino lysine and guanidino groups, as given for the original amino acids, do not necessarily apply to these groups when built into peptide

chains. Instead, by the proximity of other groups, particularly carboxyls, the basic character of lysine is markedly diminished, as data of Edsall and Blanchard show (24). The influence of the environment of the protein groups cannot be ignored, as especially emphasized by Cohn *et al.* (25, 26) and also mentioned by Highberger and Salcedo (7). The decrease in pK of the groups of the original basic amino acids built into the chain would explain their interaction with formaldehyde in a pH range a few units lower than that calculated on the assumption that the values of pK of these protein groups are the same as those of the original amino acids (9). The present author is well aware of the results of the recent investigation of the titration curve of gelatin (Lichtenstein (27)), exhibiting the effect of the removal of lysine amino groups in the titration curve at such high pH values as may be expected from the pK of the ϵ -amino group of lysine. Cohn and Edsall (26) infer from this work that deamination appears to have little effect in the acid range on the ionization of the carboxyl groups of gelatin. According to their view, it may mean that the ϵ -amino groups of lysine are too far from the carboxyls to permit the discharge of lysine groups to affect the dissociation of carboxyl groups. The behavior of solutions of gelatin evidently is not applicable to the insoluble, organized protein collagen, probably on account of different ionic environment of the interior of the two proteins.

Another possible explanation for the pH curve of the collagen-formaldehyde interaction related to the electrochemical state of collagen has previously been advanced (9). Since the total number of basic groups is about 1.0 milliequivalent and that of acid groups only 0.6 milliequivalent per gm. of collagen, an excess of 0.4 milliequivalent of basic groups is present in collagen (20, 21). At the isoelectric point (and mainly in the isoelectric range corresponding to pH 5 to 7), the electric neutrality of the system requires the surplus portion of the basic groups to be present in an *uncharged* state. The identity of the lysine amino groups with these uncharged groups has been indicated (9). The concept of the presence of free, uncharged lysine groups is in harmony with the reaction rate at pH 4 to 7 and also with the mechanism of the deamination of collagen. Furthermore, experimental proof of the participation of arginine residues in formaldehyde fixation at such relatively moderate pH values as 8 to 9 has been advanced by Highberger (6-7). However, this type of reaction does not result in stabilization of the collagen structure (9, 10) as judged by its hydrothermal stability and resistance towards swelling agents and proteinases.

Evidently, the theoretical deductions of Theis are rather speculative. The views advanced by Highberger and by the writer are in harmony with the available experimental data.

Errors due to the inclusion of solution in the pressed stock are likely to

invalidate the experiments on the location of the isoelectric point of limed hide at pH 7.6 to 7.8, reported by Theis and Jacoby (3). The isoelectric point of native collagen has been established to be in the pH range mentioned by a number of investigators (28-30). It has long been known that the isoelectric point of collagen is shifted towards the acid side (usually to pH 5 to 6) by the action of alkali (liming) (28-30).

The iodide-iodate titration method applied to pressed stock containing free, reacting solution will also explain the finding of Theis (2) that, by heat denaturation of collagen, its combining capacity for acids is markedly increased throughout the range pH 1 to 7. Since it is more difficult to press shrunk hide specimens than native hide, such samples are apt to retain more solution than native hide, which may explain the increased acid fixation. The titration curves of native and denatured hide protein, determined by pH measurements in solutions containing 2 volumes per cent of NaCl to limit swelling and by the ordinary titration method ("by difference"), are practically identical, only a slight increase being found for the H^+ fixation by denatured collagen at pH 3 to 5, which is mainly accounted for by the buffering action of solubilized protein in the heat-denatured collagen.¹ However, the increased fixation of formaldehyde by heat-denatured collagen obtained by Theis (9) is also shown in tanning at pH 8 to 13 for specimens containing only irreversibly fixed formaldehyde. This additional fixation, amounting to about 50 per cent in some instances, is mainly unaffected by heating the stock for 1 to 2 days at 105°. It has earlier been indicated that, by heat denaturation of collagen fibers, rupture of intramolecular bonds between peptide groups (H bonds) takes place. New loci for reaction are probably formed by breaking the coordinate intramolecular bonds, and the fixation of coordination-active compounds (as vegetable tannins and certain high molecular chromic salts) is greatly facilitated and increased (31). The additional formaldehyde fixed by denatured collagen is probably attached to such free, reactive groups (imino) of the peptide linkages, since any activation of basic protein groups evidently does not result in the dislocation of the collagen chains in the shrinkage reaction.

The present author recognizes the difficulties in differentiation of irreversibly and reversibly bound and unbound formaldehyde in formaldehyde-treated hide (32). The failure of the pressing method to accomplish this aim is disappointing.

SUMMARY

The pressing method for removal of unfixed formaldehyde in formaldehyde-treated collagen is shown to yield highly erratic values. The reason is that the pressed stock contains about 100 per cent of the final solution,

¹ Unpublished work by the author.

figured on the weight of protein. The solute contained in the "free water" is included in the figure for irreversibly fixed formaldehyde.

The interpretation of the formaldehyde fixation by collagen as a function of the equilibrium pH of the system, given by Theis, is criticized. His conception of the function of the imino groups of the peptide linkages as loci for the aldehyde bridge between adjacent collagen chains, leading to stabilization of the protein structure, is in conflict with experimental data. Further, his view of the attachment of formaldehyde by means of the imidazole group of histidine in the pH range of 7 to 9.5 is shown to be improbable. The important function of the ϵ -amino group of the lysine residue is pointed out.

It is further suggested that the data for the acid- and alkali-binding capacity of collagen, obtained by means of the pressing method, are misleading. This criticism also applied to experiments for location of the isoelectric point of collagen by analysis of pressed stock by means of the iodide-iodate-thiosulfate method.

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ESTIMATION OF POTASSIUM IN BIOLOGICAL MATERIALS AS POTASSIUM PHOSPHOTUNGSTATE

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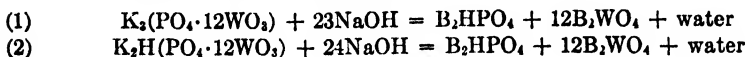
Van Slyke and Rieben (1) have recently developed a method for estimation of potassium in biological materials as potassium phosphotungstate. The low solubility of this salt in water and the high alkali requirements when it is titrated according to Toennies and Elliott (2) make the method especially suited to the estimation of small amounts of potassium with greater accuracy than had been achieved by any previous method. However, on applying it to phosphorus-rich materials such as phosphatides, it was found that the dry ashing in platinum crucibles constituted a real drawback, as P attacks platinum with formation of platinum phosphide. Under these circumstances it has been the experience of the writers that it is difficult to obtain a quantitative solution of the white ash in dilute hydrochloric acid.

To obviate this difficulty, the present method has been devised. It has the advantage that it does not require the use of platinum crucibles or a muffle furnace, and shortens the time for the complete procedure so that it can be carried through in the course of a working day. The biological material to be analyzed undergoes wet digestion with sulfuric acid and superoxol; the digest is diluted to a definite volume and treated with $\text{Ca}(\text{OH})_2$ in slight excess of the amount required to neutralize completely H_2SO_4 and to precipitate any P that may be present; CaSO_4 and $\text{Ca}_3(\text{PO}_4)_2$ formed and unchanged $\text{Ca}(\text{OH})_2$ are packed by centrifugation and an aliquot of the clear P-free alkaline supernatant is transferred to an evaporating dish and dried on a boiling water bath; this insures the removal of any ammonia present. From this point on the procedure is essentially that described by Van Slyke and Rieben (1).

The precipitate of potassium phosphotungstate obtained in the presence of a considerable amount of CaCl_2 is of different composition from that obtained by Van Slyke and Rieben's procedure. The factors used for the calculation of potassium in the present method have been derived from numerous analyses of standard solutions of potassium salts. The titration values have been plotted against the known amounts of potassium; a linear curve of the type $y = ax + b$, where y = micrograms of potassium and x

= cc. of 0.04 N NaOH, has been obtained. The gradient a has been found to be 156.0; the b value has been found to be $19.0 \pm 0.5 \gamma$ when 40 mg. of phosphotungstic acid are used and $14.0 \pm 0.5 \gamma$ when 20 mg. of phosphotungstic acid are used. 1 equivalent of K requires 10.3 equivalents of NaOH in titration.

Apparently the potassium phosphotungstate obtained in our method is not a single salt but a mixture in constant proportions of $K_3(PO_4 \cdot 12WO_3)$ and $K_2H(PO_4 \cdot 12WO_3)$. The reactions in the titrations of these two salts by alkali are respectively as follows:



in which B represents either K or Na. In the former case 7.666 equivalents of NaOH are required for 1 equivalent of K; in the latter, 12 equivalents of NaOH are required for 1 equivalent of K. It appears from these facts that the precipitate obtained in our procedure is a mixture of 45.5 per cent $K_2HPO_4 \cdot 12WO_3$ and 54.5 per cent $K_3PO_4 \cdot 12WO_3$.

The value for b does not appear to be a true solubility factor owing to solution of minute amounts of K phosphotungstate in the water used in washing. It has been found instead to be related to the amount of $Ca(OH)_2$ present in the aliquot taken for analysis ($Ca(OH)_2$ is soluble in water to the extent of 0.23 per cent) and also to the amount of phosphotungstic acid used. It appears to be independent of the amount of potassium present in the sample and, within fairly wide limits, of the volume of water used in washing.

The composition of the K phosphotungstate obtained by the original method of Van Slyke and Rieben is different from that obtained in the present method and so is the b value. By the same method of derivation used in this paper, Van Slyke and Rieben found the value for a to be 165.7 and for b to be $9 \pm 0.5 \gamma$. The value of 165.7 corresponds, within 0.4 per cent, with theoretical factors derived from an empirical formula for the precipitate of $K_2H(PO_4 \cdot 12WO_3)_2 \cdot 6H_2O$, and this composition was checked by those authors by direct analysis of the K salt obtained. The value for b in Van Slyke and Rieben's method appears to be a true solubility correction, since it corresponds to the amount of potassium phosphotungstate soluble in the volume of water used in washing.

The high value for b in the modified method makes it unsuitable for amounts of potassium below 0.08 mg. When amounts of K present in samples under analysis are smaller than this figure, the method of Van Slyke and Rieben should be used.

The method as described below has been used routinely in our laboratory for the past 2 years for analyses of phosphatides, of lipide extracts of or-

gans, of protein fractions, and of blood plasma. The results obtained have been uniformly satisfactory, double determinations checking usually within 1 per cent; *i.e.*, 4.52 and 4.54 milliequivalents of potassium for blood plasma.

Procedure

Reagents—

Sulfuric acid, concentrated; sp. gr. 1.84; reagent grade.

Superoxol, Merck, reagent grade.

Calcium hydroxide, reagent grade. It is freed of small amounts of potassium present by washing six times with distilled water and drying in the oven. It is powdered and stored in screw top bottles.

Approximately 1 *N HCl*.

4 per cent aqueous solution of *phosphotungstic acid* and *thymol blue-phenolphthalein* indicator as described by Van Slyke and Rieben (1).

Standard 0.04 *N H₂SO₄*.

CO₂-free 0.04 N NaOH. *CO₂* is removed from slightly acidified water (400 cc. + 0.25 cc. of 0.1 *N HCl* in a 500 cc. round bottom Pyrex flask) by using water pump suction for 10 minutes. 0.9 cc. of 18 *N NaOH* (3) is then pipetted into the bottom of the flask. The flask is stoppered tightly and shaken. The solution is titrated with standard 0.04 *N H₂SO₄*, with thymol blue-phenolphthalein indicator under a current of *CO₂-free air*.

Apparatus—

Digestion tubes. 16 × 125 mm. Pyrex ignition tubes, calibrated at 5 cc. volume (No. 9860 Pyrex catalogue).

Stirring rods, 3 × 160 mm.; flat footed.

Stirring rods, 1 × 150 mm., prepared as described by Salit (4).

15 cc. *centrifuge tubes* and *siphoning apparatus* for washing potassium phosphotungstate precipitate, as described by Van Slyke and Rieben (1).

Alundum bits, prepared as described by Van Slyke and Folch (5).

Micro burners.

Soda lime tubes.

Glass spoon, calibrated to hold 200 mg. of *Ca(OH)₂*; prepared as described by Van Slyke and Folch (5).

Titration apparatus, for titrating without interference from atmospheric *CO₂* (6).

Estimation of Potassium in Blood Plasma

Digestion—Plasma or serum can be digested with or without previous drying. If the analysis is to be run in a single day's work, digestion without previous drying is convenient. On the other hand previous drying has the advantage of making the digestion procedure easier and shorter.

Digestion with Previous Drying—A 1 cc. aliquot of plasma or serum is measured into a digestion tube and the tube is put in the desiccator, standing up in a beaker. The desiccator is evacuated to about 50 mm. of Hg pressure. After $\frac{1}{2}$ hour the evacuation is resumed up to the highest vacuum given by a water pump. The drying of plasma is completed overnight.

The tube with the dry plasma or serum sample is removed from the desiccator and 0.1 cc. of concentrated H_2SO_4 and two bits of alundum are added. The tube is placed in a 400 cc. beaker two-thirds full of concentrated H_2SO_4 and the H_2SO_4 bath is heated with a flame of such a size that the bath reaches a temperature of 150° in about 10 minutes. By that time the dry plasma has blackened and liquefied under the action of the hot H_2SO_4 . 4 drops of superoxol are added to the digest, 1 drop at a time. The water from the superoxol boils off rapidly. As soon as boiling decreases and blackening reappears, more superoxol is added dropwise, 3 or 4 drops at a time. The procedure is repeated a number of times (usually about ten) until blackening reappears at an obviously slower rate. Throughout the addition of superoxol the temperature of the bath is maintained between 150 – 160° . The whole process of adding superoxol usually takes about 20 minutes. This digestion in a concentrated H_2SO_4 bath can be conveniently run on twelve tubes at a time, six tubes in one 400 cc. beaker, two beakers being run at the same time.

The flame is shut off, and the tube is removed from the bath, rinsed with water on the outside, and wiped dry. Next the tube is placed on a digestion shelf and heated on a micro burner. In about 1 minute white fumes and deep blackening appear. The tube is removed from the digestion shelf, held tilted at about a 30° angle, and 1 or 2 drops of superoxol are added in such a way that the superoxol runs down along the inner wall of the tube. Superoxol clears the digest and the tube is replaced on the digestion shelf. White fumes and blackening reappear as soon as the water from the superoxol is boiled off. The addition of superoxol in the form described is repeated for as long as blackening occurs. 1 or 2 minutes are usually necessary between additions, and three or four additions usually suffice. The digestion is continued for 5 minutes after white fumes without blackening appear. The whole digestion procedure as described takes about 45 minutes for a single tube from the time of addition of concentrated H_2SO_4 to the dry plasma up to the time the digestion is completed. Twelve tubes can be conveniently run at one time and digestion completed in 60 to 70 minutes for all twelve.

Digestion without Previous Drying—1 cc. of plasma or serum is measured into a digestion tube and to it are added in succession two bits of alundum and 0.1 cc. of concentrated H_2SO_4 . The contents of the tube are thoroughly mixed by twirling. The tube is placed in a 400 cc. beaker two-thirds full

of H_2SO_4 , and the H_2SO_4 bath is heated on a gas burner to 97–99°. The size of the flame is then reduced so that the temperature of the bath increases at the rate of about 1° a minute. The contents of the tube begin to boil and the proteins that have been precipitated by addition of H_2SO_4 start to climb inside the tube. This climbing movement stops when the clump of precipitated material breaks and allows free passage to the steam. The precipitated material seldom climbs more than $\frac{3}{4}$ of an inch above the bottom of the tube. Boiling continues and blackening of the precipitated material starts. After about 10 minutes, when the temperature of the bath has reached 110°, the size of the flame is increased so that the temperature of the bath rises about 3° per minute. In another 10 to 12 minutes when the bath has reached a temperature of about 150°, the precipitated material, which has become increasingly black, is liquefied and the clumps that had climbed up the wall have fallen back to the bottom of the tube. When boiling of the liquefied material slows down perceptibly, addition of superoxol is started. The addition of superoxol is carried out as for the digestion of previously dried plasma, and from there on the procedure is exactly as described above. Complete digestion takes about 60 minutes for one tube and twelve tubes can be digested at one time in about 90 minutes.

Elimination of Phosphorus—To the cool, clear digest, water is added up to the 5 cc. mark. 200 mg. of $\text{Ca}(\text{OH})_2$ are then added. This is more than enough to neutralize the sulfuric acid present. With the 3 mm. \times 16 cm. flat footed stirring rod the mixture is stirred continuously for 5 minutes; there is no splashing if the foot of the rod is kept below the surface of the liquid. If a small rack is used, as many as twelve tubes can be stirred at one time. The tube is covered with a rubber cap and spun in the centrifuge for about 15 minutes, at 2000 R.P.M. The CaSO_4 and $\text{Ca}_3(\text{PO}_4)_2$ collect at the bottom, leaving a clear supernatant solution.

Precipitation of Potassium—3 cc. of the clear supernatant are measured into a 30 cc. Pyrex evaporating dish, which is placed on a square of chicken wire over a vigorously boiling steam bath until the solution has evaporated to dryness. This takes about 20 to 25 minutes. It insures the elimination of any ammonia that may be present. The evaporating dish is removed from over the steam bath, let cool, and 1 cc. of N HCl is added to the residue. The residue goes easily into solution, except for a thin rim of undissolved material. It is necessary to dissolve the dry residue completely. In order to do that the evaporating dish is replaced on the chicken wire, removed after a few seconds, and shaken gently. The warm N HCl will then dissolve the residue completely. Next a 4 per cent solution of phosphotungstic acid in water is added dropwise, 0.5 cc. being added (20 mg. of phosphotungstic acid) for amounts of K between 0.08 and 0.8

mg. and 1 cc. being added for amounts of K up to 1.6 mg. The solution is mixed and is again evaporated to dryness on the steam bath and removed when the odor of HCl is no longer detectable. This takes about 15 minutes.

** Transfer and Washing of Potassium Phosphotungstate*—The procedure is essentially that followed by Van Slyke and Rieben (1). The evaporating dish is removed from over the water bath and let cool. The edge of the pouring lip is greased with vaseline and 2 cc. of water are pipetted into the dish. The residue is suspended by gentle shaking and the suspension poured into a 15 cc. centrifuge tube. Without moving the dish from its position over the tube, as much of the precipitate in the evaporating dish as possible is transferred into the centrifuge tube by a stream of 4 cc. of water delivered from a Mohr pipette. Some precipitate still remains in the evaporating dish. In order to transfer it quantitatively 2 cc. of water are measured into the evaporating dish and the remaining precipitate rubbed free from the glass surface into suspension in the water by the use of a rubber-tipped rod. This suspension is poured into the centrifuge tube. This last step of the procedure is repeated twice more, 2 cc. of water being used each time. This makes a total volume of 12 cc. of water that is used for the transfer. The precipitate is washed as described by Van Slyke and Rieben (1).

Titration of K Phosphotungstate—The potassium phosphotungstate is titrated as described by Van Slyke and Rieben (1). Addition of alundum is not necessary, since the bubbling of CO₂-free air insures smooth boiling.

Blank Determination—Blank analyses are run in which water replaces the sample. In our experience the blank has been consistently zero. When there is cigarette smoke in the room, a blank value can be found which is small and constant for each set of analyses. This may be due either to small amounts of potassium or of ammonia present in the cigarette smoke (7). The value for the blank is (cc. of 0.04 N NaOH used) - (cc. of 0.04 N H₂SO₄ used).

Calculation—If 20 mg. of phosphotungstic acid have been used, the formula for calculating the amount of potassium in the sample analyzed is the following: $(0.156(T - B) + 0.014)1.677 = \text{mg. of K}$, where T stands for (cc. of 0.04 N NaOH used) - (cc. of 0.04 N H₂SO₄ used) and B is the blank value. If 40 mg. of phosphotungstic acid have been used, the formula for calculation is as follows: $(0.156(T - B) + 0.019)1.677 = \text{mg. of K}$.

When Ca(OH)₂ reacts with 0.1 cc. of H₂SO₄, 0.06 cc. of water is formed. Therefore, the volume of the neutralized digest from which the 3 cc. aliquot is taken for precipitation of potassium by phosphotungstic acid would be 5.06. It has been found, though, that in the course of stirring the Ca(OH)₂, with the digest 0.03 cc. of water evaporates, leaving a final volume of 5.03

cc. The factor 1.677 used in the calculation formula is the quotient of the ratio 5.03/3.00.

*Estimation of Potassium in Lipide Preparations or Lipide
Extracts of Organs*

Digestion—If a lipide extract is to be analyzed, an aliquot large enough to contain more than 0.08 mg. of potassium is measured into a digestion tube. The solvent is removed as follows: Two or three bits of alundum are added to the contents of the tube. The tube is placed in a beaker filled to a depth of 1 inch with concentrated sulfuric acid and the beaker placed on the boiling water bath. When the solvent has been completely boiled off, as is shown by lack of solvent smell, the samples are ready for digestion. If the material to be analyzed is a dry preparation of lipide material, a sample containing a minimum of 0.08 mg. of potassium is weighed into a digestion tube. From here on the procedure is that used for dried blood plasma.

EXPERIMENTAL

Analysis of Standard Potassium Solutions—Aliquots of standard potassium solutions containing between 0.08 and 0.8 mg. of K were analyzed under the conditions in which 20 mg. of phosphotungstic acid (0.5 cc. of 4 per cent solution) are used. The results of analyses are given in Table I. Aliquots containing between 0.17 and 1.5 mg. were analyzed under the conditions in which 40 mg. of phosphotungstic acid are used. The results are given in Table II. Linear curves of the type $y = ax + b$ were obtained, where y = micrograms of potassium and x = cc. of 0.04 N NaOH solutions. The gradient, a , was found to be 156.0 in both sets of analyses. The value for b was found to be $14 \pm 0.5 \gamma$ when 20 mg. of phosphotungstic acid were used and $19 \pm 0.5 \gamma$ when 40 mg. of phosphotungstic acid were used.

Recovery of Potassium Added to Plasma—Determinations of recovery of potassium added to plasma were run in triplicate on five different serums. The results obtained are given in Table III.

Recovery of Potassium Added to Phosphatide Preparations—Results obtained on two experiments of this type are given in Table IV.

Non-Interference of Sodium, Calcium, and Magnesium—To test this point, analyses were run on aliquots of a solution containing 20 mg. of sodium as sodium chloride and 20 mg. of magnesium as magnesium sulfate. The presence of these ions did not increase the value obtained for the blank. Analysis of standard solutions of potassium to which sodium and magnesium salts had been added in the amounts mentioned above gave exactly the same titration value as that in Tables I and II.

That calcium ions do not interfere is obvious, since the precipitation of

TABLE I
Analysis of Standard Potassium Solutions*

Potassium present† (k)	0.04 N NaOH required (T - B)	Potassium found‡ (f)	Per cent deviation from theoretical $\frac{100(f - k)}{k}$
mg.	cc.	mg.	
0.0594	0.289	0.0591	-0.5
0.0594	0.290	0.0592	-0.3
0.0682	0.351	0.0688	+0.9
0.0682	0.356	0.0696	+2.0
0.1000	0.536	0.0978	-2.2
0.1004	0.531	0.1000	-0.4
0.1092	0.595	0.1068	-2.1
0.1187	0.677	0.1196	+0.8
0.1187	0.677	0.1196	+0.8
0.1363	0.793	0.1377	+1.1
0.1363	0.797	0.1384	+1.5
0.2000	1.183	0.1985	-0.7
0.2008	1.196	0.2005	-0.1
0.2044	1.237	0.2070	+1.5
0.2184	1.318	0.2207	+1.0
0.2726	1.684	0.2765	+1.4
0.3000	1.827	0.2990	-0.3
0.3040	1.868	0.3053	+0.4
0.3040	1.865	0.3048	+0.3
0.3276	1.976	0.3222	-1.6
0.3276	1.998	0.3258	-0.6
0.3407	2.089	0.3398	-0.2
0.4000	2.484	0.4015	+0.4
0.4016	2.461	0.3980	-0.9
0.4089	2.515	0.4063	-0.6
0.4368	2.688	0.4330	-0.8
0.4777	2.969	0.4770	0.0
0.5020	3.131	0.5025	+0.1
0.5020	3.131	0.5025	+0.1
0.5460	3.408	0.5455	-0.1

* 20 mg. of phosphotungstic acid used.

† Potassium present in a 3 cc. aliquot from the H_2SO_4 digest after treatment with $Ca(OH)_2$.

‡ Calculated as mg. of K = $0.156(T - B) + 0.014$ (explanation in the text).

TABLE II
Analysis of Standard Potassium Solutions*

Potassium present† (<i>k</i>)	(0.04 N NaOH) - (0.04 N H ₂ SO ₄) (<i>T</i> - <i>B</i>)	Potassium found‡ (<i>f</i>)	Per cent deviation from theoretical $\frac{100(f - k)}{k}$
mg.	cc.	mg.	
0.1724	1.003	0.1755	+1.8
0.1724	1.008	0.1763	+2.3
0.1945	1.100	0.1906	-2.0
0.1945	1.105	0.1914	-1.6
0.1970	1.112	0.1925	-2.4
0.1970	1.152	0.1987	+0.9
0.2917	1.718	0.2870	-1.1
0.2917	1.734	0.2895	-0.8
0.2955	1.734	0.2895	-2.0
0.2955	1.734	0.2895	-2.0
0.3448	2.084	0.3441	-0.2
0.3448	2.096	0.3459	+0.3
0.3579	2.117	0.3492	-2.4
0.3579	2.172	0.3578	0.0
0.3890	2.389	0.3917	+0.8
0.3890	2.376	0.3896	+0.1
0.3940	2.432	0.3984	+1.1
0.3940	2.362	0.3874	-1.6
0.5172	3.185	0.5158	-0.3
0.5172	3.226	0.5222	+1.0
0.5834	3.598	0.5903	+1.2
0.5834	3.586	0.5784	-0.8
0.5909	3.609	0.5820	-1.5
0.5909	3.679	0.5929	+0.3
0.6896	4.266	0.6845	-0.9
0.6896	4.294	0.6888	-0.1
0.7779	4.918	0.7862	+1.1
0.7879	4.973	0.7947	+0.7
0.7879	4.977	0.7954	+0.9
0.8882	5.631	0.8974	+1.0
0.8882	5.574	0.8885	0.0

* 40 mg. of phosphotungstic acid used.

† Potassium present in a 3 cc. aliquot of the supernatant from the H₂SO₄ digest after treatment with Ca(OH)₂.

‡ Calculated as mg. of K = 0.156(*T* - *B*) + 0.019 (explanation in the text).

TABLE III
Recovery of K Added to Plasma

Sample	K originally present in plasma	K added	Titration ($T - B$) ^a	K found (f)	K calculated (c)	Error $\frac{100(f - c)}{c}$
<i>cc.</i>	γ	γ	<i>cc</i>	γ	γ	<i>per cent</i>
1.0	176.3	87.2	0.914	262.6	263.5	-0.3
1.0			0.908	261.0		-0.9
1.0			0.909	261.2		-0.8
1.0	177.3	93.2	0.934	268.0	270.5	-0.9
1.0			0.934	268.0		-0.9
1.0			0.941	269.0		-0.3
1.0	163.5	93.2	0.894	257.4	256.7	+0.4
1.0			0.891	256.5		-0.1
1.0			0.893	257.0		+0.1
0.5	89.3	99.0	0.63	188.1	188.3	-0.1
0.5			0.636	189.7		+0.7
0.5			0.628	187.8		-0.3
0.5	92.6	99.0	0.64	190.9	191.6	-0.4
0.5			0.646	192.5		+0.5
0.5			0.643	191.5		-0.1

^a $T - B = (\text{cc. of } 0.04 \text{ N NaOH}) - (\text{cc. of } 0.04 \text{ N H}_2\text{SO}_4).$

TABLE IV
Recovery of Potassium Added to Phosphatide Preparations

Brain phosphatidyl serine	Weight of sample analyzed	K present in sample	K added	K found (f)	K calculated (c)	Per cent deviation from theoretical $\frac{100(f - c)}{c}$
	<i>mg.</i>	γ	γ	γ	γ	
2.93% K [*]	5.864	172.0	254.8	431.0	426.8	+1.0
	5.456	159.7	254.8	415.2	414.5	+0.2
	5.814	170.4	254.8	421.2	425.2	-1.0
Ash-free; 0.06% K [†]	56.0	33.6	509.6	539.2	543.2	-0.8
	49.8	29.9	509.6	541.3	539.3	+0.3
	52.2	31.3	509.6	538.3	540.9	-0.5

^{*} Obtained according to Folch (8).

[†] Obtained from the preceding by treatment with 0.1 N HCl followed by dialysis and lyophilization.

potassium by phosphotungstic acid in this method takes place in the presence of relatively large amounts of calcium chloride.

In order to test whether the removal of H_2SO_4 and H_3PO_4 by $\text{Ca}(\text{OH})_2$ entrains some of the potassium present, aliquots of standard solutions of potassium were added to the 3 cc. aliquot of the supernatant used in blank determinations. The titration value was the same as that obtained in the analysis of standard solutions given in Tables I and II.

SUMMARY

1. A modification of Van Slyke and Rieben's (1) method for estimation of potassium especially suited to phosphorus-rich materials is described. The modified procedure does not require the use of platinum crucibles or of a muffle furnace. The method is suited for amounts of potassium as low as 0.08 mg.

2. The material to be analyzed undergoes wet digestion with H_2SO_4 and superoxol. H_2SO_4 and H_3PO_4 are eliminated by treatment with $\text{Ca}(\text{OH})_2$. An aliquot of the supernatant is dried, which eliminates ammonia. The residue is dissolved in N HCl and the potassium is precipitated by phosphotungstic acid.

3. The K phosphotungstate obtained in this procedure has a different composition than that obtained by Van Slyke and Rieben's (1) method. It appears to be a mixture of 54.5 per cent $\text{K}_3(\text{PO}_4 \cdot 12\text{WO}_3)$ and 45.5 per cent $\text{K}_2\text{H}(\text{PO}_4 \cdot 12\text{WO}_3)$. 1 equivalent of K as K phosphotungstate uses 10.3 equivalents of NaOH on titration according to Toennies and Elliott (2).

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MICROANALYSIS OF FRACTIONS OF A CUBIC MILLIMETER OF GAS

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The method here described permits the sampling, transfer, and determination of respiratory gases in approximately 0.35 c.mm. samples with errors not greater than ± 0.2 volume per cent and in 0.07 c.mm. samples with errors not greater than ± 0.5 volume per cent. It will handle up to 100 per cent absorbable gases. The analysis takes 4 to 6 minutes.

The method is useful in general when only a minute quantity of gas can be used for analysis. This may involve cases in which it is desirable to analyze the composition or tension of gas occurring in an extremely small place, or cases in which one wishes to study the gas tensions in tissues or fluids by introducing a minute gas bubble for equilibration.

The micrometer burette, as applied to this method of analysis, utilizes the same principle as in micrometer burettes described earlier (1-3); namely, the displacement of mercury by means of a spindle which through a mercury-tight bearing freely moves into a mercury-filled burette chamber. The fact that the burette delivers without drainage error is a principal reason for its high accuracy. This burette utilizes a 0.304 mm. steel music wire as a micro spindle and displaces 0.0726 c.mm. per mm. This figure was determined both by calculating the volume of the spindle from its diameter and by weighing the total mercury delivered by 5 times 5 mm. advance of the spindle. If we designate the volume of 0.001 mg. ($= 1 \gamma$) of water as 1 γ -liter, the smallest division on the micrometer ($= 0.01$ mm.) corresponds to 0.726 γ -liter ($= 0.0007$ c.mm.). So far the " γ -burette" has been used only in the analysis of respiratory gases and blood gases (see the next paper). It can undoubtedly be used for other gases by selecting suitable absorbents and, with slight modification, for ultramicro titrations, in connection with a calibrated micro mercury pipette (see below).

For other volumetric micro gas analytical procedures the reader is referred to papers by Krogh (4, 5), Scholander (2, 6), and Berg (7).

Principle—The gas is sampled by means of a micro mercury pipette and is stored behind mercury in a glass transfer cup. From this cup it is drawn into a micrometer burette of approximately 1 c.mm. capacity. The gas sample is measured and extruded into a series of vials containing acid and fluids for the absorption of CO_2 and O_2 . The gas menisci are located by

viewing them through a dissecting microscope. All volumes are read on the micrometer in terms of micrometer units.

Apparatus—

Micrometer burette unit. This part consists essentially of the micrometer burette itself and its holder.

Details of the construction will be seen in Fig. 1. The micrometer (A) is adapted in the following way. The frame is machined off, and the spindle cut off at the beginning of the threads (B). A holder (C) for the new micro spindle (D) is press-fitted into B and a straight piece of plain or, better,

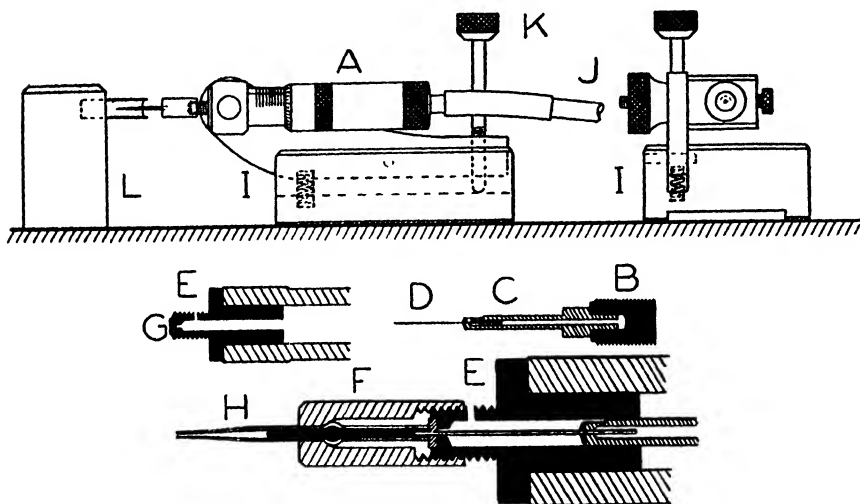


FIG. 1. Micrometer burette of 1 c.mm. capacity. A, micrometer; B, micrometer screw; C, holder for micro spindle; D, micro spindle; E, steel plug pressed into spindle bearing; F, plastic needle yoke; G, fiber disk with hole for micro spindle; H, burette needle; I, micrometer block; J and K, screws for adjusting tilt of burette; L, reagent block with vials for mercury, acid, NaOH, oxygen absorber, and water.

stainless steel music wire 0.012 inch in diameter (gage No. 3) is carefully aligned and soldered exactly in the center of C by means of a jig. The tip of D is ground to a blunt point. A steel plug (E) is loosely press-fitted into the spindle bearing. It is threaded with a 10/24 screw for the plastic needle yoke (F) and has a recess for the fiber bearing disk (G) through which the micro spindle D moves. In order to prevent pressure from being built up behind the fiber bearing (G), C should fit loosely into E; a side hole in E and a flat on the front end of C serve the same purpose. The fiber disk (G) is pressed out of a 1/32 inch fiber plate by a pipe punch and is drilled with a center hole by means of a jig (Fig. 2, F) with a pivot drill No. 10. A

Lucite yoke (*F*), threaded to fit onto *E*, has a bore sufficient to pass the burette needle. The burette needle is pulled to a fine long point; it is furnished with a shoulder and a flat ground base. By means of the yoke *F*, the needle is held pressed air-tight against the fiber bearing *G*. The burette is held in an adjustable steel block (*I*) constructed according to the principle used in an ordinary surface gage. The burette can be set at a desirable angle by means of *J* and can be tilted by means of *K*. The block has rubber corners cemented to its lower surface, so that it will not slip on the glass plate upon which it rests during analysis.

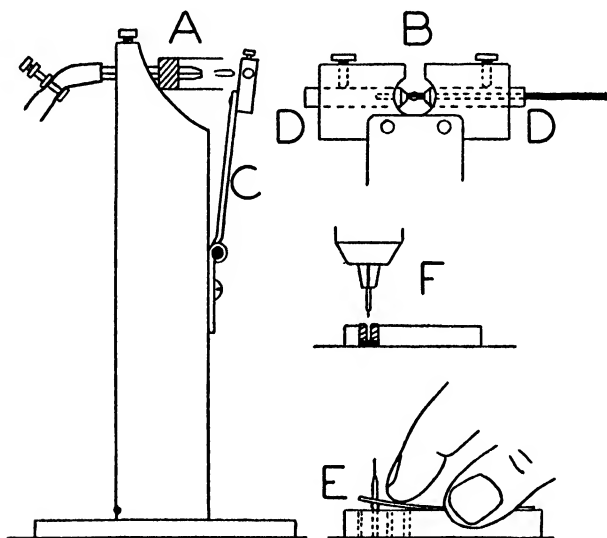


FIG. 2. Accessories. *A*, shielded micro burner; *B*, steel jig for making shoulder on burette needle; *C*, hinged support for jig; *D*, tubes with bore for needle capillary; *E*, grinding jig for needle base; *F*, jig for drilling center hole in fiber disk.

Accessories for making burette needles. A micro gas flame is set up on a wooden stand, Fig. 2, *A*. The flame is shielded from draught by means of a glass tube pressed over a piece of split rubber tubing. The steel jig (*B*) is fastened to a hinge (*C*), which is loosely screwed onto the stand. The jig is bored to receive metal tubes (*D*) which are bored for the capillary tubing; the one to the right is bored all the way through, the one to the left has a hole 8 mm. deep. The two tubes (*D*) are held by set screws with their tapered ends about 4 mm. apart. The capillary is cut straight by rotating it against a carboloy cutting tool. It is stuck through the tubing *D* until it hits the bottom of the bore. The capillary is rapidly turned while it is being carefully moved towards the flame and is slightly pushed together

to form a shoulder. The tip is pulled by free-hand about a cm. from the shoulder by moving the tubing to the flame until it becomes plastic, removing it from the flame, and pulling the ends apart to a 1 cm.-long constriction. To cut this constriction rotate the capillary against the carboly tool.

The base end of the needle must be ground flat and straight. This is done in a jig (*E*). A series of holes of slightly increasing diameter is drilled through a piece of bakelite. The needle is stuck through the tightest fitting hole. A thin sheet of perforated plastic is slipped over the needle tip and onto the shoulder. The needle base is ground flat on the finest of emery paper placed on a glass plate in the way shown by Fig. 2. Apply only light pressure. The needle is cleaned by sucking through it nitric acid, water, and acetone. It is dried and the base end is flame-polished in order to

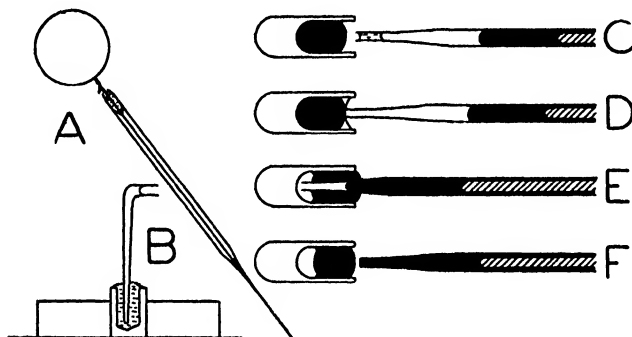


FIG. 3. *A*, mercury micro pipette; *B*, transfer cup loosely mounted in grease well; *C* to *F*, stages in loading transfer cup with gas sample from mercury micro pipette; the music wire piston is shaded.

eliminate sharp corners which would cut the steel spindle wire. With some experience it is also possible to flame-polish the tip. The bore of the needle should be large enough to slip freely onto the music wire and the bore of the tip should be fine enough to give a noticeable change in the mercury meniscus for each 0.002 mm. movement of the micrometer screw.

Transfer pipette. A piece of No. 3 music wire is bent to a 1 inch loop (Fig. 3, *A*) and the free tip is ground to a blunt point. A thin capillary tubing is selected into which the wire will fit snugly. The upper end of the capillary is blown to a chamber for grease. At the tip end a thin walled bubble is blown which is drawn out into a thin walled fine long tip. The capillary part of the pipette is filled with mercury and the grease chamber is filled from a syringe with medium Nevastane grease. The music wire is inserted through the grease into the capillary and shoved down until 0.5 cm. from

the tip constriction. The wire must fit tightly enough to hold the mercury in any position at the tip of the pipette. The pipette construction described makes it easy to handle even the most minute quantities of gases or liquids.

Transfer cup (Fig. 3, B). A thin walled glass tube of 2 to 2.5 mm. bore is fused together to form a rod with a cup on the end. The cup must have a smooth rounded bottom. The shank of the transfer cup is placed in a well of heavy Nevastane grease in which it will easily move. The total height should be such that the cup opening corresponds to the burette tip. This flexible mounting of the transfer cup insures that the pipette or burette tip will not be broken during the transfer procedure.

Block for carrying solutions. The construction of this block is seen in Fig. 1, L. A wooden or plastic block is provided with a row of 5 holes which receive the vials for mercury, acid, absorbing fluids, and acid rinsing water. The vials are approximately 7×25 mm. The first one for mercury has the opening slightly fused together; the others are coated with Clarite in toluene, around the edges, so as to hold the fluids.

Solutions—The principal considerations for the composition of the solutions are that they must wet glass well in order to give a sensitive meniscus in the fine capillary tip of the burette, and must have a low solubility for gases. If possible, the acid and alkali should have the same capillarity. The acid and alkaline sodium citrates, among a large number of solutions tried, have proved to be satisfactory. They give a sensitive meniscus and, with the burette needle moved over from one solution to the other, give identical readings for CO_2 -free gas samples and cause no bubbles or disturbing heat production. The solubility of air in the acid citrate solution is 12.5 per cent that of water, and of the alkaline citrate it is 11.5 per cent that of water. A concentrated lithium chloride solution (7), although its gas solubility can be made very low, is strongly hydrophobic and will not give a meniscus sensitive enough for accurate results in our method.

Acid solution. Dissolve 8.5 gm. of sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) in 12 cc. of water and add 0.3 gm. of citric acid. Store in a screw-capped vial at ambient air pressure.

CO_2 absorber. Dissolve 8.5 gm. of sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) in 15 cc. of water and add 0.5 gm. of NaOH . Store in a screw-capped vial at ambient air pressure.

Oxygen absorber. Solution A, mix 100 cc. of H_2O and 6 gm. of KOH ; store in a wide mouthed vessel.

Powder A, add 20 gm. of fresh sodium hydrosulfite $\text{Na}_2\text{S}_2\text{O}_4$ to 0.1 gm. of sodium anthraquinone- β -sulfonate; mix thoroughly in a mortar; store in a stoppered vessel.

Solution B, in a 5.5 cc. vial place 5 cc. of Solution A and add 0.6 gm. of Powder A; close with a finger immediately and dissolve anaerobically under

a hot water faucet, cool to room temperature under a cold water faucet, and draw with the least possible air contact the lower three-fourths of it into a 5 cc. syringe fitted with a short gage No. 20 needle with the tip cut off straight. Equilibrate with nitrogen at atmospheric pressure by leaving a small bubble in the syringe. This solution is the same as that used in the 0.5 cc. gas analyzer (6).

Mercury. c.p. mercury cleaned in a nitric acid cleaner and stored in a 1 cc. tuberculin syringe furnished with a short gage No. 20 steel needle with the end cut off straight.

Grease. Nevastane, light, medium, and heavy, made by the Keystone Lubricating Company, Philadelphia, is recommended. Store the light grease in a 1 cc. tuberculin syringe with a No. 20 gage needle cut off straight.

The acid solution, the CO₂ absorber, and the oxygen absorber are delivered during the analyses from 5 cc. syringes provided with short gage No. 20 steel needles, with the ends cut off straight. Since the acid and the CO₂ absorber dry and crystallize readily in the needle tip, these syringes should be placed with the needle tips under mercury, each in a separate vial, set in 2 holes in a block of wood. A fourth syringe with water completes the outfit.

Before analysis fill the five vials of the block in succession with mercury, acid, CO₂ absorber, O₂ absorber, and water plus 1 drop of acid solution.

Assembling Burette—Screw the micrometer spindle all the way out and grease the micrometer screw lightly with light Nevastane. With a thin rod push the fiber washer out from its socket. Insert the spindle and advance it until the micro spindle (Fig. 1, *D*) projects 0.5 cm. out of the nozzle (Fig. 1, *E*). Grease the spindle with light Nevastane from the syringe and, while twisting the spindle back and forth, gently slip on the fiber bearing, push it back, and gently press it into position. Screw the micro spindle back until it projects 2 mm. and cover it completely with a drop of light Nevastane.

Fill the burette needle with mercury from the syringe by holding the base of the burette needle against the flat opening of the syringe needle. When the burette needle is full, close the tip end with a finger and cover the base end of the needle completely with a drop of light Nevastane, without trapping any air bubble. The grease drops are joined together, and the needle is now fitted over the projecting micro spindle, upon which it will hang. The grease seal effectively prevents air from getting into the burette. The slight amount of grease which enters with the spindle does no harm. The yoke is slipped on and tightened gently.

Test the assembly for tightness by screwing the mercury out to the tip opening. If the mercury does not pop out or in when going through the tip opening, the assembly is satisfactory. If it does, there is a leak or a trapped air bubble in the needle. Screw the micrometer in to zero and cut

the mercury flush with the needle tip. Draw in 5 or 10 mm. of air and bring the meniscus back to the needle tip again. The micrometer should now read zero within 0.5 division.

Transfer of Gas Sample to Analyzer—In some cases it can be arranged that the burette needle can be directly introduced to the gas source through a drop of mercury or acid solution. In such cases the micrometer is brought slightly past zero (see below) with the mercury meniscus flush with the needle tip. The tip is introduced through the acid or mercury seal, and the sample drawn in and sealed with the acid or mercury when withdrawn. Usually the transfer pipette and transfer cup will have to be used. The clean and dry transfer cup is filled from the bottom with mercury delivered from the tuberculin syringe. Inspect under the binocular to make sure that no air is trapped in the cup. With the mercury extended to the tip of the transfer pipette a gas sample is drawn into the pipette, followed by a seal

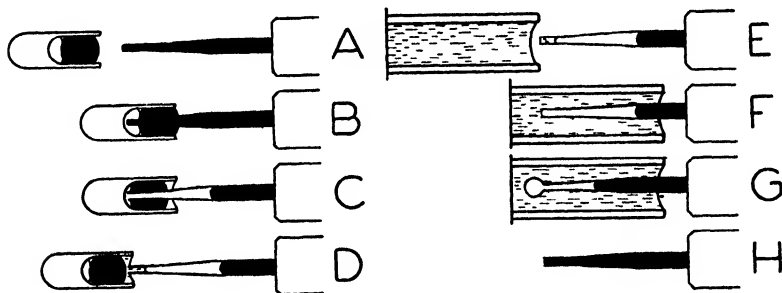


FIG. 4. A through D, drawing sample into burette needle from transfer cup; E through H, stages in analyzing gas sample in the vials of the reagent block.

of liquid in equilibration with the gas, or of acid solution, or mercury. The sample is transferred to the mercury-filled transfer cup, according to Fig. 3, C, D, E, F. This is easily done free-hand, if the pipette tip is being slid along the lower cup edge diagonally upwards through the mercury.

The transfer cup, containing enough gas for a duplicate analysis, is now placed in front of the burette tip and a sample transferred to the burette according to Fig. 4, A, B, C, D. At A the micrometer is at approximately 1.5 divisions past zero, with the mercury flush with the tip. At B the mercury seal has been pierced centrally. At this point, the mercury content of the burette must be so adjusted that the mercury meniscus in the tip is exactly flush with the needle opening when the micrometer reads zero. If this is not so, pull the tip back into the mercury and readjust. At C the gas sample has been drawn into the burette and a sealing drop of acid has been added to the cup. At D the cup is withdrawn and the burette tip is automatically sealed by the acid.

Analysis—The analytical procedure is seen in Fig. 4, *E, F, G, H*. At *E* the vial with the acid is advanced to the burette tip, where the sealing drop is extended to be flush with the tip opening. At *F* the acid has been moved in over the needle tip and the meniscus has been located to a reading position, which is exactly flush with the needle's opening. The total gas volume must be well inside the fluid to insure temperature control. The

TABLE I
Comparison between Analyses on 0.5 Cc. Analyzer and Analyses on γ -Burette

0.5 cc. samples Per cent			0.370-0.362 c.mm. samples Per cent							
Room air and gas mixtures										
CO ₂	0.06-0.10		0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.1
O ₂	20.86-20.90		20.8	20.9	20.8	20.8	20.9	20.9	20.9	20.8
N ₂	79.00-79.06		79.0	79.0	79.2	79.1	79.1	79.1	79.1	79.1
CO ₂	5.00	5.01	4.9	4.9	4.9	4.9	4.8	4.8	5.0	4.8
O ₂	15.72 15.73		15.7	15.7	15.8	15.9	15.8	15.8	15.8	15.8
N ₂	79.28 79.25		79.4	79.4	79.3	79.2	79.4	79.4	79.2	79.4
CO ₂	15.38 15.36		15.2	15.1	15.3	15.2	15.2	15.3	15.3	15.4
O ₂	4.97	4.99	5.0	5.0	5.0	4.9	4.9	5.1	4.9	4.9
N ₂	79.65 79.64		79.9	79.9	79.7	79.9	79.9	79.6	79.8	79.7
CO ₂	29.53 29.53		29.5	29.5	29.5	29.4	29.2	29.4	29.5	29.4
O ₂	50.86 50.85		50.5	50.7	50.6	50.5	50.6	50.7	50.5	50.8
N ₂	19.61 19.62		20.0	19.9	19.9	20.2	20.2	19.9	20.0	19.8
CO ₂	83.59 83.59		83.4	83.3	83.3	83.5	83.5	83.5	83.5	83.3
O ₂	15.17 15.20		15.4	15.5	15.3	15.5	15.4	15.3	15.4	15.2
N ₂	1.23	1.21	1.2	1.3	1.3	1.1	1.1	1.2	1.0	1.4
Tank oxygen										
CO ₂	0	0	0	0.1						
O ₂	99.63 99.65		99.4	99.4						
N ₂	0.37	0.35	0.6	0.6						

All analyses are consecutive. The first sample is room air taken directly with the burette. All the others involve complete transfer with a transfer pipette and a transfer cup. Usually duplicates or triplicates have been taken from the cup. It is apparent from the table that the accuracy throughout the whole range can be expected to be ± 0.2 per cent, or often better.

micrometer is read, V_1 . The NaOH solution is now moved onto the needle and most of the gas is moved out into the solution (*G*) and the micrometer turned slightly back and forth a couple of times until absorption is complete. After only a few seconds the meniscus can be relocated to the tip (*F*), and the reading V_2 is taken.

Next, the vial with the oxygen absorber is moved onto the needle, and most of the gas is extruded into it while the block is being moved slightly

back and forth. When absorption is complete, the meniscus is relocated as in *F*, and the dilute acid of the last vial is brought over the needle. Without the gas being extruded, the needle tip is washed a couple of times until all the red oxygen absorbent has been washed away, after which the final reading, V_3 , is taken in vial No. 2, containing the concentrated acid solution.

The gas is then expelled in the acid wash of vial No. 5, and the needle tip is rinsed a few times for the next analysis. The acid wash is then expelled, and the burette tip is dried outside and inside by holding a suction

TABLE II

Comparison between Analyses on 0.5 Cc. Analyzer and Analyses on γ -Burette

0.5 cc. samples Per cent		0.075-0.069 c.mm. samples Per cent			
CO ₂	0.06- 0.10	0.1	0.0	0.1	0.0
O ₂	20.86-20.90	20.7	20.9	20.9	20.8
N ₂	79.00-79.06	79.2	79.1	79.0	79.1
CO ₂	5.00 5.01	4.9	5.2	4.5	4.9
O ₂	15.72 15.73	15.7	15.2	16.3	15.2
N ₂	79.28 79.25	79.4	79.6	79.2	79.9
CO ₂	15.38 15.36	14.7	15.0	14.5	15.3
O ₂	4.97 4.99	5.5	5.4	5.2	5.1
N ₂	79.65 79.64	79.8	79.5	80.2	79.6
CO ₂	29.53 29.53	29.6	29.2	29.4	29.5
O ₂	50.86 50.85	50.5	50.8	51.2	50.7
N ₂	19.61 19.62	19.9	20.0	19.4	19.8
CO ₂	83.59 83.59	83.9	83.0	83.7	83.5
O ₂	15.17 15.20	14.9	15.5	15.5	15.5
N ₂	1.23 1.21	1.2	1.5	0.8	1.0

All samples are consecutive. The first sample is room air taken directly with the burette. All the others involve complete transfer with a transfer pipette and transfer cup. Usually duplicates were taken from the cup. It is apparent from the table that an accuracy of ± 0.5 per cent can be obtained throughout the whole range.

tube close to it. With the mercury meniscus flush with the needle tip, the micrometer should now read zero within 1 division; if it does not, re-adjust the mercury content of the burette in vial No. 1.

Calculation of Results.

$$\text{Volume \% CO}_2, \text{ dry,} = \frac{V_1 - V_2}{V_1} \times 100$$

$$\text{Volume \% O}_2, \text{ dry,} = \frac{V_2 - V_3}{V_1} \times 100$$

$$\text{Volume \% N}_2, \text{ dry,} = \frac{V_3}{V_1} \times 100$$

Accuracy of Method—The accuracy of the method was tested by comparing it with results obtained with the 0.5 cc. analyzer (6).

Forty-two analyses of gas mixtures in samples of approximately 0.35 c.mm. covering a wide range of compositions, are presented in Table I. In the first twenty-four analyses listed of gases approaching the composition of respiratory gases, sixty-one out of seventy-two determinations with the micro analyzer differed by 0.1 per cent or less from the standard values. In the remaining eighteen analyses of gases of unusual composition, forty-seven out of fifty-four determinations differed by 0.2 per cent or less from the standard values.

In Table II are given the results of twenty analyses in samples of 69.0 to 75.0 γ -liters (0.069 to 0.075 c.mm.). Out of the sixty determinations, fifty-five came out within ± 0.5 per cent of the standard values.

We are greatly indebted to Dr. Laurence Irving for his constant encouragement and advice, to Mr. Walter Flagg for most valuable assistance and for most of the analyses with 0.07 c.mm. samples, and to Mr. Otto Hebel for his skill and craftsmanship in making the instruments and in designing many of the constructional details and mechanical features of the instrument.

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SUMMARY

A method is described which permits the sampling, transfer, and volumetric analysis of respiratory gases in 0.3 c.mm. samples with errors not greater than ± 0.2 volume per cent and in 0.07 c.mm. samples with errors not greater than ± 0.5 volume per cent. It will handle up to 100 per cent absorbable gases. The analysis takes 4 to 6 minutes.

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MICRO BLOOD GAS ANALYSIS IN FRACTIONS OF A CUBIC MILLIMETER OF BLOOD

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Analysis of fluids in minute amounts or from sharply localized regions permits definition in corresponding detail of respiratory gradients and quantities. The method here described permits sampling, transfer, and analysis of 0.7 to 0.14 c.mm. blood samples for carbon dioxide and oxygen with an accuracy of ± 0.6 per cent for the larger samples and ± 1.5 per cent for the smaller samples. Nitrogen determinations are less accurate. The analysis takes 15 to 20 minutes.

Principle—The blood is sampled into a fine glass capillary and transferred directly to a mercury-filled micrometer burette needle of 1 c.mm. capacity, accurate to approximately 1 in 3000. Between the burette needle and the micrometer is interposed a mercury-filled detachable extraction tube which on its walls has a coating of dried acid and ferricyanide. The tip of the needle, containing the blood sample, is capped with wax, and the needle-extraction tube unit is detached from the micrometer and placed in a centrifuge tube with the lower end of the extraction tube dipping into the mercury. By the force of centrifugation, the mercury falls from the needle into the extraction tube, the blood dissolves the reagents, and the gases are released into the vacuum space. Reabsorption of the extracted blood gases is prevented by suddenly stopping the centrifuge, whereby the rising mercury shoots past the blood and reagent solution, sticking to the walls of the tube, leaving the extracted gas in contact only with mercury. The burette needle is detached from the extraction tube and put onto the micrometer burette. The wax tip is removed in a drop of acid solution and the gases are analyzed as described in the preceding paper (1).

Apparatus—The micrometer burette used is the same as that described in the foregoing paper (1). It is necessary for this method that the micro plunger be made of stainless steel. If plain steel is used, lactic acid in the reagents will produce hydrogen bubbles in the burette needle and spoil the analysis. For the analysis of blood or other liquids the following additions are necessary.

The *extraction tube* (Fig. 1, A) is made from a 1.5 mm. bore glass tube. The shoulder is prepared with the jig used for micro gas analysis (1). The burette end of the tube is ground flat and straight in a jig (Fig. 2, C, D,

and *E* (1)), and flame-polished. The neck must be round and must fit the burette needle well (Fig. 1, *A*). The extraction tube is held against the micrometer fiber disk by means of a steel yoke (*B*) which threads onto the micrometer and engages the shoulder of the extraction tube, as did the plastic yoke used previously (the preceding paper). The yoke is made by drilling a steel rod and filing the sides away, the base end then being threaded and split.

Oven for melting wax (Fig. 1, *C*). A brass tube is furnished with ventilation holes and a side tube and is slipped over a small 110 volt light bulb connected with a rheostat. A small vial with beeswax resting on a rod above the light bulb is kept heated to just above the melting point of the

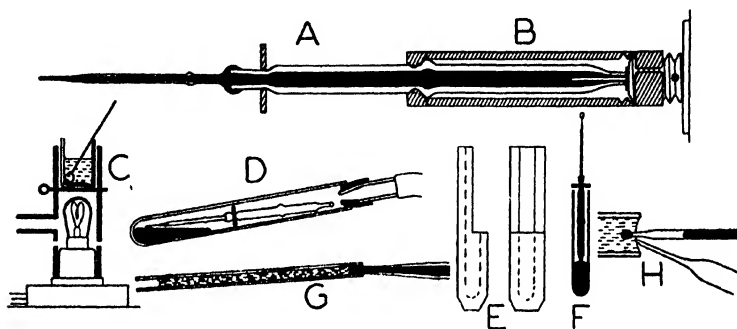


FIG. 1. *A*, extraction tube with sealed on burette needle. *B*, steel yoke holding extraction chamber tight against fiber bearing for micro plunger; *C*, lamp for keeping wax melted and for drying reagents in extraction tube; *D*, evacuation of extraction tube with attached needle; *E*, wooden holders for centrifuge; *F*, centrifuge tube with extraction chamber unit ready for centrifugation; *G*, transfer of blood sample by means of capillary hung on to the tip of the burette needle; *H*, cutting off wax cap in acid with drawn out, broken off glass rod.

wax. A steel wire bent to a small loop is left in the vial and used for applying the melted wax. The side tube serves as a support for the extraction tube when the reagents are being dried onto its walls.

High vacuum pump. A Cenco Hyvac or similar pump is connected with a glass tube large enough to contain the extraction tube with the burette needle attached, Fig. 1, *D*.

Angle centrifuge. A small light type is preferable. Wooden adapters for the centrifuge tube are prepared, which will fit into the metal tubes of the centrifuge (Fig. 1, *E*). It is essential to be able to stop the centrifuge suddenly. For this purpose a board 30×20 cm. with a hole 12 cm. in diameter is clad with felt around the lower edge of the hole. By pressing the board gently over the spinning conical housing of the centrifuge it can

easily be brought to a stop at a controlled rate without damage to the centrifuge.

Tool for removing wax cap. A 2 mm. glass rod is drawn out to a fine rod (Fig. 1, *H*), which is broken off to form a sharp edge. It serves for removing the wax cap of the burette needle.

Cleaning rods. A piece of wire which will easily slip through the extraction tube is sealed onto a piece of glass tubing for easier handling. A similar tool is prepared with a very fine wire for cleaning the burette needle.

Blood sampling pipettes. A glass tube is drawn out to a thin walled capillary just big enough to fit over the burette needle tip. If the tissue has to be speared to get the sample, one end of the capillary is drawn out to a tip. In many cases the mercury transfer pipette is preferable ((1) Fig. 3, *A*).

Solutions—In addition to the syringes with acid, water, absorbing solutions, mercury, and grease, as described for the gas analysis method (see the previous paper), the following five solutions are necessary. These solutions are kept in five wide mouthed 50 cc. vials which are placed in a row of 5 holes drilled in a block of wood.

Acid ferricyanide. Add 15 cc. of water to 5 cc. of concentrated lactic acid (85 per cent) in a small measuring cylinder and pour into a mortar. Add 2 gm. of potassium ferricyanide and dissolve in the mortar. Store in a wide mouthed vial.

Urea solution. Dissolve 40 gm. of urea in 100 cc. of water. Store part of it in a wide mouthed vial.

In addition, store water, carbon tetrachloride, and acetone, each in a wide mouthed vial.

Procedure

Assembling of Burette—The extraction chamber, detached from the burette needle, is cleaned by sucking it full of the urea solution and rinsing it mechanically with the wire tool. Carbon tetrachloride and acetone aid in getting rid of the wax. With the micrometer end of the tube attached to the suction line, draw water through the tube, followed by acid ferricyanide solution. Then place the tube immediately into the side arm of the oven so as to produce a more or less continuous coating of dry acid ferricyanide on the inside of the tube. Clean the burette needle in the same way as described for the extraction tube, using the finer wire tool. Stick the base of the needle in the neck of the extraction tube and apply wax around the joint by means of the wax loop. Heat the joint very cautiously until the wax melts and flows in around the needle and fills out the corner between the ground needle base and the extraction chamber.

Care is required so that the needle is not plugged with wax. Seal the rear end of the extraction tube superficially by depositing quickly a button of wax on it, which can easily be pushed off. Stick the unit, tip down, in a tube containing some mercury (Fig. 1, *D*) and connect with a vacuum pump. Tilt the tube so that the needle tip is not blocked by the mercury. After $\frac{1}{2}$ to 1 minute's evacuation, turn the tube vertically so that the tip is below the mercury surface, stop the pump, and open slowly to air. The unit will then completely fill with mercury. Remove it from the tube, and, handling it by the rubber collar to prevent undue heating, close the tip with a finger and push the wax button off with a fingernail. If the tube is not completely full of mercury, fill in the remainder by means of the mercury pipette ((1) Fig. 3, *A*), which is stuck into the rear opening. Apply a small drop of grease over the rear end of the tube, and likewise over the micro spindle, which is advanced 1 to 2 mm. out of the fiber bearing. Slip the yoke over the extraction chamber and join the extraction chamber onto the micrometer. The grease drops on both of the joining parts prevent trapping of air bubbles. Screw the micrometer in to zero, leaving the needle tip in mercury, which occupies the first vial in the reagent block. Due to the large volume of the burette it is quite sensitive to temperature, and must be allowed to equilibrate a couple of minutes before the mercury will remain stationary at the needle tip.

Transfer of Blood Sample—The blood is sampled directly in a fine, freshly drawn capillary tube just fine enough to slip over the burette needle tip (Fig. 1, *G*). The sampling end of the capillary is either blunt or drawn out to a fine tip which is cut off before transfer. If blood cannot be drawn by either of these direct methods, the mercury pipette used for gas sampling (see the preceding paper) is used and the capillary tubing is charged from it. By means of the mercury drop on the reagent block adjust the mercury flush with the needle tip when the micrometer is set at 0 mm. With the blood sample out to the end of the transfer capillary place the latter suspended on the needle tip (Fig. 1, *G*), suck off any blood which may have oozed out around the junction, and draw in 10 mm. of blood measured with the micrometer. The surface tension at the junction prevents any air from entering the needle or capillary. Remove transfer capillary and apply wax from the wire loop on the needle tip by several light touches until it forms a pear-shaped cap. This forms the seal for the vacuum extraction to follow. Unscrew the yoke and detach the extraction chamber unit. Refill the rear end of the extraction tube with mercury, using the mercury pipette until the mercury protrudes as a small drop. An added security against trapping air around the opening is to moisten it with a little saliva on the finger.

Vacuum Extraction—Drop the extraction tube into the small centrifuge

tube which contains just enough mercury to cover the drawn out open end of the tube (Fig. 1, *F*). Further descent of the tube is prevented by the rubber ring around the neck of the extraction tube. Place the centrifuge tube in the wooden adapter (*E*) which goes into the angle centrifuge. Run the centrifuge up to full speed. This throws the mercury in the extraction tube down close to the mercury level of the centrifuge tube, creating a vacuum space for the extraction (2). On its way down the blood dissolves the acid ferricyanide from the walls. The centrifuge is slowly stopped, letting the blood solution back up into the needle in order to mix with the traces of blood which remain on the walls. The centrifuge is then run up to full speed for 1 minute to complete the extraction. The brake board is placed over the conical centrifuge housing and pressed down gently, stopping the centrifuge in about 1 second. With a little experience the proper braking time will be found which will make the mercury shoot past the blood which adheres more firmly to the walls of the extraction tube, leaving the extracted gas trapped by mercury in a practically dry needle tip. This inversion effectively prevents reabsorption of the extracted gases. Without lifting the extraction tube out of the mercury, inspect under the dissecting microscope to make sure that inversion is complete; that is, that no blood solution is left on top of the mercury meniscus and that no gas bubbles are trapped along the walls and no blood remains in the tip. Lift the tube out and pull the burette needle out of the extraction chamber neck with a straight pull. Refill if necessary the rear end of the needle with mercury from the pipette until the mercury protrudes. Cover the rear end with a drop of grease from the syringe and do likewise with the micro spindle which protrudes about 3 mm. from the fiber disk. Join the grease drops of the needle and plunger together and shove the needle onto the plunger until it hits the fiber disk. No air bubble should be trapped in this procedure. Now slip the plastic yoke used for micro gas analysis (see the previous paper) over the needle and tighten up gently. The gas is now under positive pressure. Relieve this by screwing the micrometer back about one and a half to two turns. Move the reagent block (see (1)) so that the acid solution surrounds the needle tip.

Gas Analysis—Remove the wax cap by cutting it off on an oblique towards the tip (Fig. 1, *H*). Transverse poking may break the needle tip. If the wax is cut right at the tip of the needle, it will open up as a flat lid and will not trap gas as it may if it is poked off as a cup. As soon as the wax cap breaks open, the acid meniscus will move into the needle. The meniscus is immediately brought out to a reading position flush with the tip and the micrometer is read (see the previous paper, Fig. 4, *F*). The partial pressure of carbon dioxide is generally very high, and some absorption into the acid will take place if this step is delayed.

The rest of the analysis follows the description in the preceding paper.

Calculations—In addition to the factors for converting the gas volumes to s.t.p., two more factors enter the calculations; namely, a capillarity factor and a factor for incomplete extraction of dissolved gases. Both are determined empirically.

Capillarity Factor—This depends upon the fact that the gas volumes are compressed above the ambient pressure by capillary forces in the needle tip. This compression varies with each needle and to a slight degree with the wetting properties of the different solutions. The capillarity factor is determined for each new needle once and for all in the following way. Attach the needle directly to the micrometer without the extraction chamber and set the micrometer at zero with the mercury at the tip of the needle. Draw in exactly 10 mm. of air and place the vial with the acid over the tip. Adjust the meniscus flush with the tip and read. The difference of, e.g., 25.0 (= 0.25 mm.) divisions represents the compression at the reading point by capillary force, amounting in this example to 2.5 per cent. Hence the factor by which all readings of gas volumes with this particular needle must be corrected is 1.025. The factor is smaller for large bore tips, larger for small bore tips.

Factor for Incomplete Extraction of Gases The amount of gas which is not removed from the solution by extraction (corresponding to the *I* factor in the Van Slyke manometric techniques) is determined empirically by the following procedure.¹ A blood sample of known composition is extracted in the centrifuge and inverted as in a regular analysis. The whole unit is put back onto the micrometer, and the wax cap cut off dry. The mercury is brought to the tip of the needle, ejecting the extracted gas, and the needle is wax-capped again without trapping any air. The unit is extracted in the centrifuge and analyzed again in the regular manner. If 1000.0 divisions of blood on first extraction yield 500.0 divisions of CO₂ and on second extraction yield 25.0 divisions of CO₂ (= 5 per cent of 500), 5 per cent would still have been unextracted. The total unextracted will therefore be 25.0×1.05 , or close to 26, which equals 5.1 per cent of 500.0, and the final factor is 1.051. The combined factor for capillarity (e.g., 1.03) and unextracted CO₂ (e.g., 1.051) is then $1.051 \times 1.03 = 1.082$; similarly, for oxygen the capillarity factor remains the same, but the reabsorption factor is much less, e.g., 1.005; the combined factor for oxygen hence is $1.03 \times 1.005 = 1.035$.

The general formula for calculation of the gas volumes can be written as

¹ This factor undoubtedly varies with the temperature, but between 20–25°, not enough apparently to be empirically determined by the method of the second extraction.

follows, according to derivation from Van Slyke's and Stadie's formula (3) p. 249),

$$V = \frac{B - W}{760} \times F_t \times F_i \times F_c$$

where V = gas volume (S.T.P.) in terms of micrometer divisions; B = barometric pressure; W = water vapor tension of blood solution (a close

TABLE I
Temperature Correction Factor F_t *

°C.		°C.		°C.	
15	0.9455	20	0.9286	25	0.9124
16	0.9421	21	0.9255	26	0.9092
17	0.9382	22	0.9221	27	0.9060
18	0.9353	23	0.9188	28	0.9029
19	0.9320	24	0.9156	29	0.8998

* Adapted from Peters and Van Slyke, Table 27, p. 260 (3).

TABLE II
Comparison between Analyses of Mammalian Blood Gases on Van Slyke Apparatus
and on γ -Burette

		1 cc. samples Vol. per cent			0.72 c.mm. samples Vol. per cent			
Oxygenated (W. F.)	CO ₂	28.3	28.3	28.3	28.3	28.7	28.4	28.2
	O ₂	19.0	19.5	19.2	18.7	18.5	19.1	18.6
	N ₂	1.5	1.6	1.6	2.2	1.9	1.8	2.0
Venous stasis (W. F.)	CO ₂	55.2	55.1		53.9	55.0	55.3	55.4
	O ₂	6.0	6.4	6.1	5.9	5.9	5.8	5.9
	N ₂	1.2	1.6		2.6	2.8	1.9	2.7
Drowned rat	CO ₂	55.2	55.1		54.8	54.7	54.6	55.4
	O ₂	0.5	0.5		0.3	0.3	0.3	0.2
	N ₂	1.3	1.2		1.7	1.8	1.7	1.4

Out of twelve CO₂ determinations, ten are inside of ± 0.5 per cent of the true figure. Out of twelve oxygen determinations, ten are inside ± 0.5 per cent of the true value. All nitrogen values are too high by 0.2 to 1.6 per cent.

enough value for W is obtained by subtracting 5 from the temperature; at 20° it is close enough to 15 mm., at 25° to 20 mm., etc.); F_t = the factor for temperature (Table I); F_i = the factor for incomplete extraction; F_c = the factor for capillarity.

The final formula for calculating the volumes per cent of gases in a sample of blood measured by 10 mm. on the micrometer is accordingly $V/10 = (B - W)/760 \times F_t \times F_i \times F_c$ where V is the gas volume (CO₂, O₂, CO, N₂) in terms of micrometer units.

Accuracy of Analysis—The accuracy of the carbon dioxide, oxygen, and nitrogen determination has been checked against the Van Slyke-Neill

TABLE III
Comparison between Analyses of Avian and Fish Blood Gases on Van Slyke Apparatus and on γ -Burette

	1 cc. samples				0.6 c.mm. samples							
	Vol. per cent				Vol. per cent							
Duck, venous	CO ₂	44.4	44.4	45.1	44.3	46.0	46.0	45.3	44.9	44.8	44.4	
	O ₂	16.3	16.2	15.5	15.4	15.8	15.3	15.2	15.2	14.8	15.8	
	N ₂				2.0	1.8	1.5	1.3	1.4	1.6	2.7	
Carp, partly aerated	CO ₂	10.8	10.9		11.1	10.7	11.0	10.8				
	O ₂	11.3	11.3		10.8	10.4	10.6	10.6				
	N ₂				3.0	3.3	2.7	3.5				
Carp, aerated	CO ₂	6.6	6.4		6.3	6.5	6.4	6.1				
	O ₂	16.4	16.2		16.7	16.1	16.1	16.3				
	N ₂				5.8	3.2	2.9	5.3				

Out of fifteen CO₂ determinations in duck and carp blood thirteen are within ± 0.5 per cent of the true value. Out of fifteen oxygen determinations in duck and carp blood twelve are inside ± 0.7 per cent of the true value. Most nitrogen values are from 1 to 2 per cent too high.

TABLE IV
Comparison between Analyses of Blood Gases on Van Slyke Apparatus and on γ -Burette with Extremely Small Samples

	1 cc. samples				0.14 c.mm. samples							
	Vol. per cent				Vol. per cent							
Venous stasis (W. F.)	CO ₂	52.4	52.6		50.8	51.3	50.5	54.0	52.7	51.7		
	O ₂	4.3	4.2		5.2	5.2	4.3	5.7	4.8	5.0		
	N ₂	1.8	1.9		4.6	5.1	3.8	2.9	4.3	4.5		
Rabbit, arterial	CO ₂	41.1	41.1		40.7	42.0	36.6	39.7	41.3			
	O ₂	15.2	15.1		15.5	13.2	15.9	15.9	15.9			
	N ₂	1.2	1.1		3.1	4.3	6.6	4.5	4.9			
Rabbit, arterial	CO ₂	41.5	41.5		41.1	40.5	41.9	41.8				
	O ₂	15.9	15.5	15.4	16.1	16.3	17.1	16.4				
	N ₂	0.9	1.2		5.6	5.8	8.2	5.5				
Duck, venous	CO ₂	50.7	51.2		49.7	53.1	49.9	51.2				
	O ₂	9.2	8.9		8.5	8.0	8.1	7.2				
	N ₂	0.9	1.3		2.8	3.9	7.1	3.4				

Out of nineteen CO₂ determinations fifteen are inside ± 1.5 per cent of the true value. Out of nineteen oxygen determinations fifteen are inside ± 1 per cent of the true value. All nitrogen values are from 1 to 6 per cent too high.

manometric method by the analyses of different samples of mammalian, avian, and fish blood. The results are seen in Tables II, III, and IV.

It is believed that the method is quite generally applicable to a variety of different body and other liquids encountered in experimental physiology, or in dealing with other solutions. If the liquid contains no proteins, or if it is too low in proteins to adhere to the walls of the extraction tube when the centrifuge is stopped, the reagents which are dried on the extraction chamber may be made sufficiently adhesive to hold by adding aquaresin (Glyco Products Company, Inc., New York). By this means the dissolved gases in water or in crustacean blood have been successfully determined. It has been our experience that the analysis of true solutions can be considerably more accurate than the analysis of blood.

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SUMMARY

A method is described which permits the sampling, transfer, and micro gasometric analysis of 0.7 to 0.14 c.mm. blood samples for carbon dioxide and oxygen with an accuracy of ± 0.6 per cent for the larger samples and ± 1.5 per cent for the smaller samples. Nitrogen determinations are less accurate. The analysis takes 15 to 20 minutes.

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COMPARISON OF A MODIFIED FOLIN PHOTOMETRIC PROCEDURE AND THE NINHYDRIN MANOMETRIC METHOD FOR THE DETERMINATION OF AMINO ACID NITROGEN IN PLASMA

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Evolution of CO_2 by reaction of α -amino acids with ninhydrin is characteristic of the groups $\text{C}(\text{NH}_2)\text{COOH}$ and $\text{C}(\text{NH}-\text{CR})\text{COOH}$, and measurement of the evolved CO_2 affords the most accurate procedure available for the determination of free α -amino acids in biological material (1). At pH 2, used for application of the procedure to blood filtrates (2), all the amino acids known to be yielded by protein hydrolysis give quantitative yields of CO_2 except glycine and tryptophan, which give respectively 5 and 10 per cent less than theoretical, and lysine which gives about 5 per cent more ((1), Table IV). The older nitrous acid method (3) and Sørensen's (4, 5) formaldehyde titration as applied to blood filtrates include aliphatic amines other than amino acids, and proline and hydroxyproline are not determined in the nitrous acid method. The colorimetric procedure developed by Folin (6) likewise includes aliphatic amines other than amino acids (7); however, Van Slyke and Kirk (5) found that when applied to blood and urine the colorimetric method gave results different from those of the formaldehyde titration and the nitrous acid method, which showed approximate agreement with each other. Neither Van Slyke and Kirk (5) nor Re and Potick (8) could obtain by the colorimetric method even approximate recovery of added amino acids. However, a recent photometric¹ modification of this colorimetric method by Frame, Russell, and Wilhelmi (9) and Russell (10), which requires but 0.2 ml. of plasma for duplicates, is simple and rapid, and embodies improvements which, it appeared, might make the method sufficiently accurate for many purposes. The work described below was done to determine with what degree of accuracy the photometric procedure, either as described by Frame, Russell, and Wilhelmi (9, 10) or with modifications, could be used as a substitute for

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¹ The term "photometric" is here used to indicate the procedure in which a photometer measuring optical density is employed, "colorimetric" for one in which a Duboscq type colorimeter is used. The term "photometric nitrogen" describes the nitrogen determined by the photometric procedure, "ninhydrin nitrogen" the nitrogen determined by the ninhydrin- CO_2 manometric method. "Ninhydrin nitrogen" is identical with "carboxyl nitrogen" used in some previous publications.

the more laborious ninhydrin manometric method in analyses of normal and pathological human plasma. Bonsnes (11), in a paper appearing as this goes to press, finds that in normal and pregnant women the photometric method gives values "some 7 to 9 per cent higher" than the ninhydrin manometric method.

EXPERIMENTAL

Procedure

Photometric—The procedure described by Frame, Russell, and Wilhelmi (9) with the modifications recommended by Russell (10) was used. Because of high blanks, the 1,2-naphthoquinone-4-sodium sulfonate obtained commercially was purified by Folin's method (6). A standard curve of glutamic acid was used for the individual amino acids and for plasma determinations instead of a mixed standard of glycine and glutamic acid. For each set of determinations a standard curve based on at least three concentrations (usually with 4, 8, and 12 γ of glutamic acid nitrogen per 15 cc. of final colored solution) was established. The Coleman junior spectrophotometer, with wave-length scale set at 475 m μ , was used for most of the analyses of prepared amino acid solutions and all the plasma determinations. A few determinations were done on the Beckman quartz spectrophotometer to check the position of the absorption maxima observed in the Coleman instrument with individual amino acids. Tungstic acid filtrates were used.

Ninhydrin Manometric Analyses—The procedure described by Hamilton and Van Slyke (2) as modified by these authors (12) was used throughout. The results are corrected for urea nitrogen for plasmas with normal urea nitrogen. With abnormally high urea nitrogens the carboxyl tubes were incubated for 3 hours at 60° with 200 mg. of ninhydrin as described in (2).

Results

Agreement with Beer-Lambert Law—In photometric determinations made with the Coleman junior instrument the constancy of the K in the equation $D = cK$, was generally adequate, but variations of 5 per cent and higher were observed for some amino acids over the range of 4.0, 8.0, and 12.0 γ of α -amino nitrogen per 15 cc. of final colored solution. (D indicates optical density calculated as $\log_{10} (I_0/I)$, c is the concentration of amino nitrogen, K is a constant for the cuvette and wave-lengths used.) With the Beckman spectrophotometer glutamic acid gave constant K values for the amino nitrogen range 8.0 to 40.0 γ per 15 cc. of final colored solution (Table I). With concentrations of amino nitrogen below 8 γ or above 40 γ per 15 cc., deviations of several per cent appeared. At the lower concentrations

the percentage error in optical density measurement increased, and above 40 γ deviations from the Beer-Lambert law became marked. Deviations of all types were more marked with the less precise Coleman instrument than with the Beckman. With the Coleman instrument, however, the reproducibility of the standard curve of glutamic acid was adequate: in twenty determinations of 4.0, 8.0, and 12.0 γ of glutamic acid α -amino nitrogen the mean optical densities (in cylindrical cuvettes of 16 mm. bore) and estimated standard deviations were 0.110 ± 0.006 , 0.212 ± 0.004 , and 0.317 ± 0.006 respectively.

Determinations of Individual Amino Acids—Analyses were made of solutions of the amino acids listed in Table II. Solutions of each amino acid were prepared, in most cases in three concentrations, 4, 8, and 12 γ of α amino nitrogen per 15 cc. of final colored solution. The amino acid prep-

TABLE I
Variation of Molar Extinction Coefficient with Concentration of Glutamic Acid

NH ₂ -N glutamic acid per 15 cc.	Optical density	Molar extinction coefficient
γ		
4.0	0.067	3540
8.0	0.138	3654
12.0	0.206	3628
16.0	0.278	3672
20.0	0.350	3698
40.0	0.692	3656
60.0	0.997	3512
80.0	1.262	3334
100.0	1.559	3296
200.0	2.26	2388

Data obtained with Beckman spectrophotometer; $\lambda = 475 \text{ m}\mu$; slit width 0.07 mm.; depth of transmitting layer of solution, 1 cm.

arations were checked for purity by analysis for α -amino nitrogen by the manometric ninhydrin method, and when the result differed from theoretical, the observed α -amino nitrogen content was used to calculate the concentration of the solution for photometric analysis. Exceptions were cysteine, for which the theoretical nitrogen content was used, and glutamine, the purity of which was determined by the method of Hamilton (13).

The colors were developed as described by Russell (10), from duplicate portions of each amino acid solution diluted to 5 cc., and the final colored solutions were brought to a volume of 15 cc. Readings on the duplicates were made on the Coleman or Beckman photometer from 10 to 20 minutes after the final dilutions. Calculations were made from standard optical density curves prepared with glutamic acid. All densities were measured at $\lambda = 475 \text{ m}\mu$.

The results are given in Table II. Duplicate readings on solutions with the same concentration of amino acid usually agreed within 2 or 3 per cent, but solutions of different concentrations, as is seen in Table II, often differed from each other by more than 5 per cent in the amount of theoretical amino nitrogen indicated by their readings.

TABLE II

Wave-Length of Absorption Maxima and Per Cent of α -Amino Nitrogen Determined against Glutamic Acid Standard

Amino acids	Wave-length maxima	Per cent of 1 atom of N per mole determined at $\lambda = 475 m\mu$ with varying amounts of α -N present per 15 cc. solution					
		Coleman spectrophotometer			Beckman spectrophotometer		
		4 γ	8 γ	12 γ	12 γ	16 γ	20 γ
	<i>mμ</i>						
Glutamic acid. .	475	100	100	100			
Alanine	482	90	98	102	108.2	106.2	104.6
Arginine	478	83	84	86	91.2	87.4	84.3
Lysine	495	150	159	167	162.2	161.2	153.8
Glycine	490	80	88	90	93.2	91.4	89.7
Proline . .	478	60	59	58			
Hydroxyproline .	475	59	58	57			
Sarcosine	465	58	60	62			
Histidine .	478	85	93	101			
Phenylalanine	478	106	103	106			
Tyrosine	475	90	90	103			
Tryptophan .	475	99	100	99			
Valine	478	99	98	100			
Cystine . . .	478	90	86	84	81.5	80.9	78.6
Cysteine .	485	87	84	80	79.6	77.0	75.5
Aspartic acid	478	95	98	100			
Asparagine .	472	100	100	98			
Glutamine .	475	99	99	99			
Leucine	480	105	104	102			
Isoleucine .	470	103	97	100			
Norleucine	475	105	107	100			
Methionine . .	480	83	90	93			
Threonine . . .	485	92	90	90			
Serine	480	95	96	93			
Ammonia	500	80	93	94			

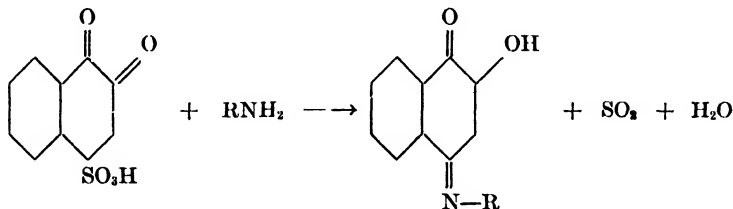
The grosser deviations from theoretical values shown in Table II, in particular the low values for proline and hydroxyproline, are similar to those found by Frame, Russell, and Wilhelmi (9) with Filter 490, although the percentage values differ somewhat from theirs.

Fading occurred in varying degree with all the amino acids. During

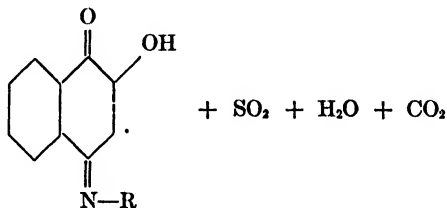
the period 10 to 20 minutes after the colored solutions were diluted fading in solutions of most of the amino acids was of the order of 1 to 2 per cent of the optical density measured 10 minutes after dilution. Fading was much more rapid in the cases of proline and hydroxyproline. As an example, in an experiment with proline, the optical density indicated 59 per cent of the theoretical nitrogen when the reading was made 10 minutes after the final dilution, and only 25 per cent when the reading was made on the same solution 30 minutes after the dilution.

Optical Densities of Solutions of Compounds of 1,2-Naphthoquinone-4-Sodium Sulfonate with Glutamic Acid and Lysine—To ascertain whether the observed differences in wave-length of maximal absorption (Table II) were not artifacts due to fading or to presence of varying amounts of unreduced naphthoquinone sulfonate the colored compounds of glutamic acid and lysine with the naphthoquinone² were prepared and the optical density curves were determined in the absence of reagents other than phthalate buffer used to set the pH. To solutions of glutamic acid and lysine, each containing 250 mg. of α -amino nitrogen in 100 cc. and adjusted to pH 9.2 by addition of 1.0 N NaOH and borate buffer, were added 500 and 1000 mg. of the naphthoquinone sulfonate. The mixtures were heated on the bath for 10 minutes, cooled, and 100 cc. of concentrated HCl were added to each

² Frame, Russell, and Wilhelmi (9) suggest that the compounds formed are analogous to that formed by the quinone with aniline



Schiller (14) showed that, with α -amino acids, the reaction occurs with evolution of a molecule of CO_2 . Hence the products to be expected from a monoamino acid, $\text{R}\cdot\text{CH}_2\cdot\text{NH}_2\cdot\text{COOH}$, would be



while each of the two NH_2 groups of lysine would presumably condense with a molecule of quinone.

solution. Precipitates formed and were separated by centrifugation. The lysine compound was washed three times with distilled water; the glutamic acid compound because of its higher solubility was washed only once. The lysine compound was purplish red in color and relatively insoluble in water. The glutamic acid compound was light red and easily soluble. Solutions in a phthalate buffer of pH 2.6 had optical density maxima at 500 $m\mu$ for the glutamic acid compound and at 475 $m\mu$ for the lysine compound. Similar differences were found at pH 1.8. The relations are the reverse of those found under the conditions of routine analysis, with reduced naphthoquinone present; the glutamic acid compound then has its maximum at 475 $m\mu$ and the lysine compound at 495 $m\mu$ (Table II). In 50 per cent ethanol there was a slight shift in wave-length of maximal density to 510 $m\mu$ for the glutamic acid compound and 490 for the lysine compound. After addition of 2 N NaOH the lysine solution turned brown with a gradually increasing optical density towards the shorter wave-lengths, while the glutamic acid solution turned green with a definite peak at 600 $m\mu$.

Comparison of Results Obtained in Plasma Analyses with Ninhydrin Manometric and Photometric Procedures—Picric acid filtrates for the ninhydrin manometric method and tungstic acid filtrates for the photometric method were made from the same samples of plasma from patients in the Hospital of the Rockefeller Institute. The results are recorded in Table III, and a distribution of the photometric values in percentile relation to the ninhydrin nitrogen determined is given in Fig. 1. Approximately 20 per cent of the photometric values are within ± 5 per cent of the ninhydrin values, but 54 per cent deviate by more than ± 15 per cent from the ninhydrin values, and some deviations exceed 400 per cent.

Recovery of Added Amino Acid Nitrogen—Plasma samples were obtained from a nephrotic child not in hypoaminoacidemic crisis, from a normal individual, and from a cirrhotic patient with jaundice. Two casein enzymatic partial hydrolysates containing some polypeptide nitrogen were used as the source of added amino acid nitrogen. Hydrolysate *a* was the commercial product, "amigen," used to treat the nephrotic patients; it contained in 1:30 dilution 20.1 mg. of total nitrogen per 100 cc. Hydrolysate *b* was a solution of 0.5 gm. of a dried casein hydrolysate in 100 cc. of distilled water; it contained 65.0 mg. of total nitrogen per 100 cc. Tungstic acid filtrates were made of each plasma and of each plasma plus an equal volume of hydrolysates. Portions of the same filtrates were used for the ninhydrin and the photometric determinations. The average recovery was 97 to 99 per cent for the ninhydrin procedure in terms of ninhydrin nitrogen, and 100 to 116 per cent for the photometric procedure in terms of photometric nitrogen. There were differences of 19 to 48 per cent between the ninhydrin nitrogen and the photometric nitrogen determined on the same filtrates (see the last column, Table IV).

TABLE III

Comparison of Concentrations of Amino Acid Nitrogen in Human Plasma Determined by Ninhydrin and Photometric Procedures

Plasma No.	Diagnosis	Ninhydrin N	Photometric N	Difference	Per cent ninhydrin nitrogen determined by photometric procedure
		(a)	(b)	(a - b)	
		<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	
1	Normal	4.25	4.2	-0.05	98.8
2		3.52	4.7	+1.18	133.5
3	Latent nephritis	4.30	4.1	-0.20	95.4
4		3.64	3.6	-0.04	98.9
5		3.96	3.9	-0.06	98.5
6		2.92	3.2	+0.28	109.6
7		3.52	3.1	-0.42	88.0
8		4.81	3.4	-1.41	70.7
9	Nephritis active	4.13	4.5	+0.37	108.9
10	Nephritis, terminal	2.37	11.3	+8.93	477
11		3.88	17.3	+13.42	446
12		3.91	19.2	+15.29	491
13	Nephrotic syndrome	3.54	4.2	+0.66	118.6
14		3.78	3.2	-0.58	84.6
15		4.09	3.0	-1.09	73.4
16		3.59	3.2	-0.39	89.1
17		3.51	3.6	+0.09	102.5
18		5.09	4.6	-0.49	90.4
19		3.73	2.8	-0.93	75.1
20		3.81	3.2	-0.61	84.0
21		6.00	5.6	-0.40	93.4
22		3.66	2.3	-1.36	62.9
23		4.85	4.8	-0.05	99.0
24		4.64	3.8	-0.84	81.9
25		3.45	2.5	-0.95	72.5
26		5.10	4.7	-0.40	92.2
27		3.12	2.9	-0.22	93.0
28		3.69	3.0	-0.69	81.3
29		3.44	5.3	+1.86	154.2
30		3.09	3.1	-0.01	100.3
31		3.17	2.9	-0.27	91.5
32		3.29	3.9	+0.61	118.6
33		3.40	2.3	-1.10	67.6
34	Nephrotic crisis	2.32	2.8	+0.48	120.6
35		2.25	1.9	-0.35	84.4
36		2.40	2.1	-0.30	87.5

TABLE III—*Concluded*

Plasma No.	Diagnosis	Ninhydrin N	Photometric N	Difference	Per cent ninhydrin nitrogen determined by photometric procedure
		(a)	(b)	(a - b)	
		<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	
37	Nephrotic ascitic fluid	4.69	3.7	-0.99	78.9
38	Hepatitis	5.03	6.0	+0.97	119.4
39		4.18	5.1	+0.92	122.0
40		7.24	7.2	-0.04	99.5
41		5.07	4.6	-0.47	90.7
42		8.27	7.5	-0.77	90.7
43		5.55	4.4	-1.15	79.3
44		5.55	4.2	-1.35	75.7
45	Hepatitis, terminal	5.54	7.8	+2.26	139.7
46	Cardiac failure	5.11	5.1	-0.01	99.9

In Plasmas 10, 11, and 12 amino nitrogen values by the nitrous acid method were 11.3, 13.1, and 15.5 mg. per cent respectively.

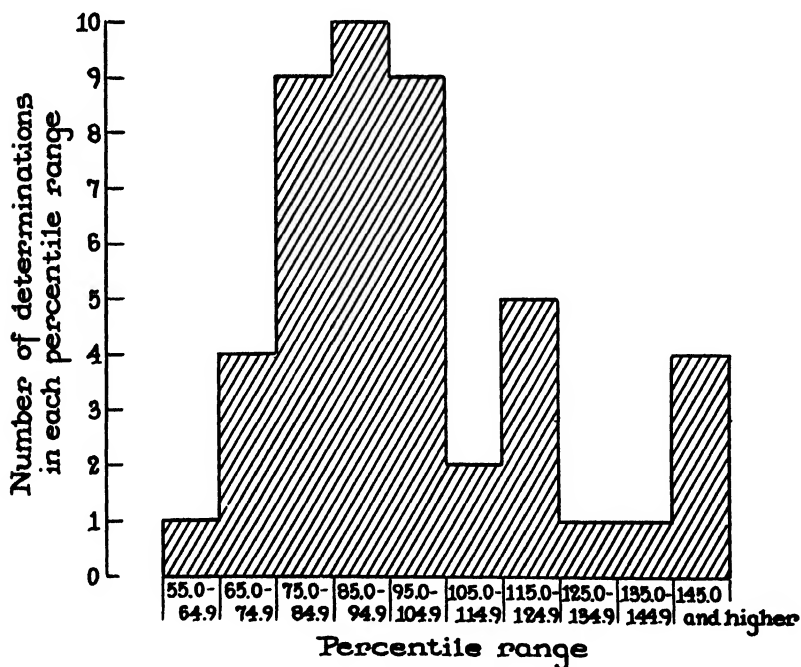


FIG. 1. Distribution of per cent of ninhydrin nitrogen determined by the photometric procedure in human plasma.

Attempts to Modify Photometric Procedure—Several attempts were made to improve the photometric procedure. The attempts failed and will therefore be only briefly reported. In the standard photometric procedure it was noted that after the naphthoquinone-amino acid solution was acidified with HCl and formaldehyde the color faded at an appreciable rate, especially in the cases of proline and hydroxyproline. Adjusting the final

TABLE IV
Recovery of Amino Nitrogen Added to Plasma As Protein Hydrolysate

Analyses of plasma and hydrolysates separately				α -Amino N in 1:1 mixtures of plasma and hydrolysate							
Material	α -Amino N found			Ninhydrin manometric			Photometric			Photometric found Manometric	
	Ninhydrin manometric	Photometric	Photometric Manometric	Calculated*	Found	Found Calculated	Calculated*	Found	Found Calculated		
	mg. per 100 cc	mg per 100 cc		mg per 100 cc.	mg per 100 cc		mg per 100 cc.	mg per 100 cc			
Plasma 1 (nephrotic)	3.17	2.14	0.675	a 7.25 b 5.58	7.13 5.40	0.983 0.968	a 7.47 b 6.91	8.48 7.98	1.135 1.156	a 1.19 b 1.48	
Plasma 2 (normal)	5.50	5.28	0.960	a 8.42 b 6.75	8.32 6.54	0.988 0.970	a 9.04 b 8.48	9.62 8.50	1.065 1.003	a 1.16 b 1.30	
Plasma 3 (cirrhotic)	7.29	8.02	1.101	a 9.32 b 7.65	9.10 7.58	0.977 0.991	a 10.41 b 9.85	11.20 10.14	1.076 1.029	a 1.23 b 1.34	
Average.						0.980			1.077	1.28	
Hydrolysate	a 11.34 b 7.99	12.80 11.67	1.129 1.460								

Rows marked *a* and *b* give results from plasma plus Hydrolysates *a* and *b* respectively.

Tungstic acid filtrates were used for all analyses, aliquots of the same filtrate being used for both photometric and manometric analysis.

For the manometric analyses, the reaction with ninhydrin was carried out at pH 2.5 with 20 mg. of ninhydrin per cc., heated for 15 minutes in the water bath.

* Calculated values are those estimated from analyses of separate plasma and hydrolysate by the method used for the mixture.

pH to 4.0 with oxalic acid, as in Hoffman's modification (15), resulted in less fading, but much higher blanks. Reducing agents other than thio-sulfate were tried in order to prevent fading of the proline and hydroxyproline compounds or to bleach them out completely. Ascorbic acid, sodium hyposulfite ($\text{Na}_2\text{S}_2\text{O}_4$), titanous trichloride, sodium bisulfite (NaHSO_3), and hydrogen sulfide were tried and failed to do either. Other quinone reagents such as anthraquinone α -sulfonate, anthraquinone

β -sulfonate, and naphthoquinone-4-thiosulfonate were tried. Preliminary tests indicated that this last compound would be as suitable as the naphthoquinone sulfonate, but it was abandoned because of its instability. Other substituted 1,2- and 1,4-naphthoquinones were also tried unsuccessfully.

DISCUSSION

Some of the amino acids do not give theoretical results by the photometric procedure even when the standard is a glutamic acid solution of the same molar concentration, and, when their concentrations are varied, not all of the amino acids obey Beer's law at the wave-length of 475 m μ . The compounds formed by the naphthoquinone with the different amino acids have different optical density peaks and considerable fading occurs in some cases. Under these circumstances it might be expected that in plasma analyses the results obtained with the photometric procedure would differ somewhat from those obtained with the more specific and quantitative ninhydrin manometric method. In fact, however, the results differ, in some cases more widely than would be anticipated from the behavior of the individual amino acids that presumably form the mixture (see Fig. 1 and Table III). The largest differences occurred with the plasma of patients in uremia in which the ninhydrin-determined nitrogen was within normal levels but the photometric nitrogen and the nitrous acid-determined nitrogen were both about 4 times as great. It appears that in such cases the photometric and nitrous acid determinations must include amino nitrogen, possibly from amines, that is not α -amino nitrogen. The high results obtained with the nitrous acid method are of the same order of magnitude as those of Kirk (16) who found, in an investigation of changes in nephritis, the amino nitrogen to be as high as 38 mg. per cent. In contrast, the routine ninhydrin manometric determinations carried out in this hospital on uremic plasmas have rarely exceeded 5 mg. per cent. On the other hand, most of the photometric determinations in the nephrotic cases are lower than the ninhydrin determinations, although in some cases the photometric values are higher.

In one important respect our results with Frame, Russell, and Wilhelmi's modification of the Folin colorimetric method are better than those reported by authors (5, 8) who tested the original Folin (6) method; *viz.*, whereas the original was found to give less than even approximate recoveries of amino acids added to blood, the present modification gives approximate recoveries. The reason is probably that in the modification of Frame *et al.* a much greater excess of naphthoquinone reagent is used than in the original Folin procedure; whereas the original requires 5 mg. of the quinone (1 cc. of 5 per cent solution) for filtrate representing 1 cc. of blood or plasma,

in the modification of Frame *et al.* the same amount of reagent is used for filtrate representing only 0.08 cc. of blood or plasma. The photometric procedure still bears the disadvantages, relative to the manometric, in that the photometric is less uniform in its results with different amino acids, suffers from fading of the color obtained with some of them, and can give results that have no relation to the amino acids if amines or other interfering substances are present.

SUMMARY

On comparison of the photometric and ninhydrin manometric methods in analyses of forty-six normal and pathological human plasmas, 54 per cent of the photometric results showed a deviation from the ninhydrin results greater than ± 15 per cent. Maximal deviations were from -37 to $+491$ per cent. The gross plus deviations of the photometric results were observed in analyses of uremic plasmas which give similar high results by the nitrous acid method. It appears that the high photometric results in these cases may be due to the presence of amino nitrogen other than α -amino acid nitrogen.

An attempt has been made to increase the accuracy of the Folin colorimetric procedure for the determination of amino acids in blood; the absorption curves yielded by the different amino acids and the variable rates of fading have been studied, and different naphthoquinones and reducing agents have been tested as reagents. However, no procedure for color development has been found better than that of Frame, Russell, and Wilhelm, as modified by Russell.

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THE POSSIBILITY OF DETECTING INDIVIDUAL PROTEINS IN BLOOD SERUM BY DIFFERENTIATION OF SOLUBILITY CURVES IN CONCENTRATED SODIUM SULFATE SOLUTIONS

II. COMPARISON OF SOLUBILITY CURVES WITH RESULTS OF ELECTROPHORESIS EXPERIMENTS*

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In earlier work (1, 2) the significance of differentiating solubility curves of the serum proteins in concentrated sodium sulfate solutions was demonstrated in order to arrive at a better understanding of these curves. The diagrams resulting from differentiation showed the existence of three individual proteins. The euglobulin and the albumin were characterized by a distinct precipitation, maximal in Na_2SO_4 concentrations of 145 and 305 gm. of Na_2SO_4 per liter respectively. The pseudoglobulin, precipitating between the two main fractions, did not show such easily definable solubility properties.

The quantities of these three proteins can be derived from the surface area of the peaks in the differentiation diagrams. It was shown that by a single precipitation by sodium sulfate solutions of 190 and 260 gm. per liter about the same results can be obtained as by the computation of the surface areas.

There was a striking resemblance between our curves and the figures obtained by electrophoresis of human sera. Evidence was obtained that euglobulin and γ -globulin, pseudoglobulin and $\alpha + \beta$ -globulin, and the two albumins are identical. It seemed desirable to check our solubility curves against electrophoretic analyses on some highly pathological human sera.

Methods

The normal serum of subject H. and the sera of subjects D. with chronic hepatitis, W. with multiple myeloma, and E. with chronic nephritis were investigated.

All blood samples were drawn in the morning while the patients were

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fasting. As it was necessary to take rather large amounts of blood, it was not possible in all instances to collect a sufficient amount without stasis. Especially in the case of subject W., who had very bad veins, stasis was inevitable. All protein values of this subject will therefore be a little too high. Nevertheless, this error will not interfere with the comparison between differentiation diagrams and electrophoresis curves.

Methods for the delineation of solubility curves have been described previously (1, 2). A simplification of the iodometric nitrogen determination of Teorell (3) was used.

TABLE I
Concentration of Sodium Sulfate Used for Titration of Proteins

Concentrations of Na ₂ SO ₄			
In solutions added	In final serum-salt mixture	In solutions added	In final serum-salt mixture
<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>
114	108	269	255
123	117	271	257
132	125	282	268
139	132	292	277
149	142	300	285
155	147	310	294
159	151	318	302
169	161	328	311
185	176	336	319
194	184	346	329
204	194	357	339
206	196	367	348
216	205	375	356
224	213	384	365
235	223	400	380
243	231		

As filtration in the incubator was much hampered by the watch-glasses covering the funnels, some filtration experiments were made with uncovered funnels in an incubator in which several large Petri dishes, filled with water, were placed. Loss of weight amounted to 0.17 per cent of the protein-salt mixture during the 1st 80 minutes of filtration, and to 0.26 per cent during the 1st 140 minutes. As filtrations are always completed within the 1st hour, errors due to evaporation were neglected in these experiments and all filtrations were made without watch-glasses.

Sodium sulfate solutions of pH 7.0 were prepared according to instructions given previously (1, 2), the concentrations varying from 400 to 100 gm. per liter. The concentrations of these solutions were checked gravimetrically in duplicate. Enough of each solution was added to 1 cc. of se-

rum to bring the volume to 20 cc. The concentrations of the solutions are presented in Table I with the Na_2SO_4 concentrations of the corresponding serum-salt mixtures. The latter are used in Figs. 1 to 4.

Electrophoresis experiments were carried out in the apparatus of Tiselius with a phosphate buffer of pH 7.6 and ionic strength 0.037 with 0.15 M NaCl. Electrophoresis diagrams were obtained by the cylindrical lens method of Philpot-Svensson (4, 5). Serum was diluted with buffer to a protein concentration of about 20 gm. per liter before dialysis. The protein concentrations were determined refractometrically. Each run was carried out for 2 to 3 hours at 0° at 200 volts and a current of 50 milliamperes. After separation of the components the diagram was photographed and the negative was enlarged several times. The area under each peak was de-

TABLE II
Comparison of Protein Fractionation by Sodium Sulfate and Electrophoresis

Serum	Total protein $\times 100$	Albumin				$\alpha + \beta$ -Globulin Pseudoglobulin				γ -Globulin		Euglobulin	
		Electrophoresis		Differential curve		Electrophoresis		Differential curve		Electrophoresis		Differential curve	
		per cent	gm. per l.	per cent	gm. per l.	per cent	gm. per l.	per cent	gm. per l.	per cent	gm. per l.	per cent	gm. per l.
H., normal..	73.0	56.0	40.9	56.6	41.4	20.4	14.9	14.8	10.8	23.6	17.2	28.5	20.8
D., hepatitis	100.4	33.9	34.0	26.4	26.5	19.3	19.4	17.5	17.6	46.8	47.0	56.1	56.3
W., myeloma	98.0	33.8	33.1	29.6	29.0	20.1	19.7	15.8	15.5	46.2	45.3	54.5	53.5
E., nephritis...	51.2	21.0	10.8	20.1	10.3	60.3	30.9	38.6	19.8	18.7	9.6	41.2	21.1
Normals from Majoor (2).	73.0			54.2	39.5			18.2	13.3			27.7	20.2
Westermann (6).		59.9				19.2				20.9			
Olhagen (15)		57.8				21.5				20.7			

finer by perpendiculars to the base line from the lowest point of the gradient curve on each side of the peak and measured by means of a planimeter. In Table II the percentages, as derived from the negative limb, are recorded. As a rule γ -globulin percentages from the positive limb are somewhat higher than those from the negative limb used here. This is probably due to the fact that the δ -boundary is not separated from the γ -globulin in the positive limb. With this apparatus and the same buffer, Westermann (6) found the following averages in fourteen determinations of normal human sera; albumin 59.9 per cent of the total protein, α -globulin 7.3 per cent, β -globulin 11.9 per cent, γ -globulin 20.9 per cent.

Observations

Our observations are recorded in Figs. 1 to 4. The upper part of each consists of the solubility curve and the corresponding differentiation dia-

gram; the lower part shows the electrophoretic analysis. In all the cases the differentiation diagram shows a distinct euglobulin peak. The albumin peak is clearly visible in normal serum and in the sera of the subjects with

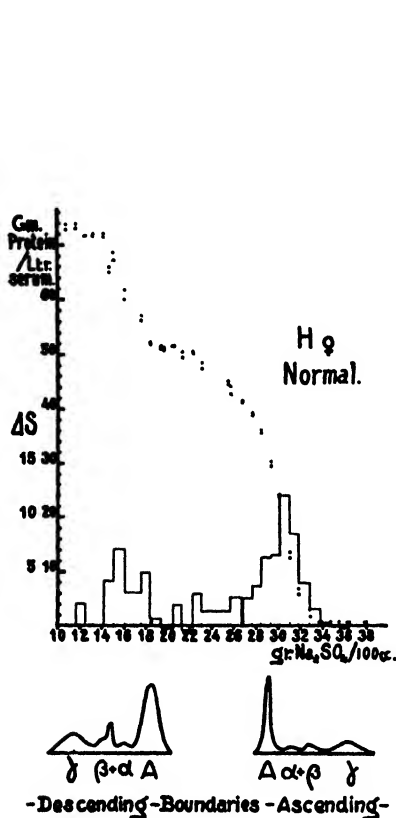


FIG. 1

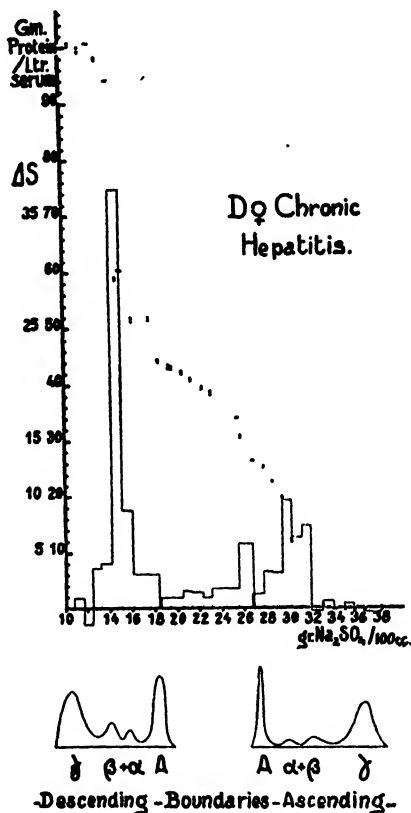


FIG. 2

FIG. 1. Differentiation of the curves and the recording in broken line graphs were made as before (1, 2). It must be admitted that these broken line graphs are mathematically not correct. They should be replaced by real curves. As the exact form of these curves cannot be computed from the restricted number of the experimentally fixed points, especially in the pseudoglobulin area, and since the arbitrary construction of such curves may lead to misinterpretation and to the construction of non-existing peaks, the broken line form has been retained.

FIG. 2. See the legend for Fig. 1

myeloma and hepatitis, but in the serum of subject E. (chronic nephritis) the albumin peak is not visible.

In Figs. 1 to 4 of this investigation and Figs. 4 and 5 of the previous one

(2), the salt concentrations at the beginning, the maximum, and the end of the euglobulin and albumin peaks were estimated as accurately as possible. The averages of these values were computed and are presented in Table III.

The salt concentration in normal as well as in highly pathological sera, at which maximal precipitation of albumin is found, is remarkably constant. The maximal precipitation of the euglobulin is in all pathological sera shifted to the left, but the difference is small.

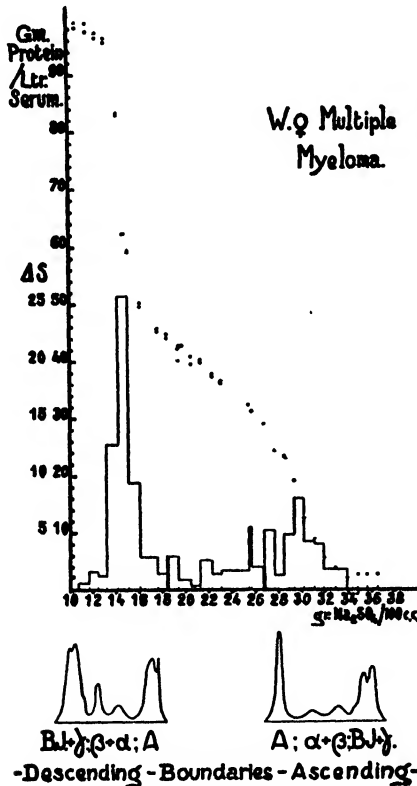


FIG. 3. See the legend for Fig. 1

In every-day clinical work, an approximately correct separation of the serum proteins by simple, single precipitation of Na_2SO_4 solutions would be very useful. From Table III and Figs. 1 to 4 it can be shown that by the use of sodium sulfate concentrations of 185 and 268 gm. per liter the serum proteins can be divided into a euglobulin, a pseudoglobulin, and an albumin fraction. It must be admitted that, by an undeniable overlapping of the three fractions in the above-mentioned salt concentrations, no absolute

separation takes place. In the euglobulin fraction some pseudoglobulin will be present and vice versa. The same holds good for the fractions ob-

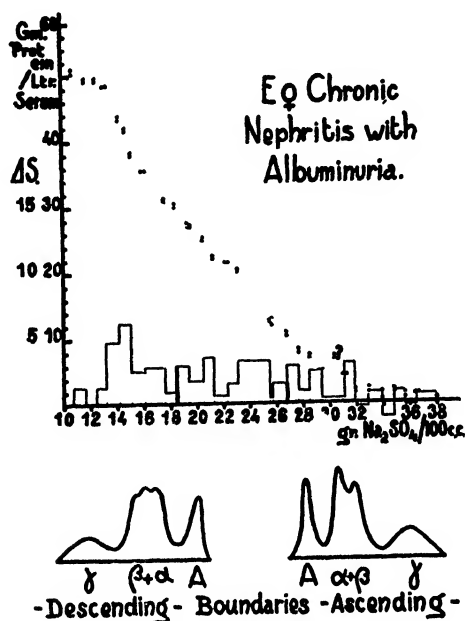


FIG. 4. See the legend for Fig. 1

TABLE III

Concentration of Na_2SO_4 in Gm. Per Liter Required to Precipitate Euglobulin and Albumin in Seven Solubility Curves

	Euglobulin			Albumin*		
	Beginning	Maximum	End	Beginning	Maximum	End
Average	113	148	185	268	306	345
Range	100-125	138-162	176-199	260-275	300-313	330-355
“ for pathological sera		138-148			300-313	
Range for normal sera		145-162			305-308	

* As in the case of the patient with nephritis, no distinct albumin peak is evident; this curve could not be used for the calculation of the salt concentration necessary for precipitation of albumin.

tained by precipitation with sodium sulfate solutions of 268 gm. per liter. From the form of the differentiation diagram, it can, however, be deduced that only small amounts of the different fractions will contaminate the

neighboring ones. The amounts of euglobulin in the pseudoglobulin fraction and of pseudoglobulin in the euglobulin fractions probably will be of about the same magnitude. For this reason the fractions obtained, though not absolutely pure, will approximate the percentages of the real proteins closely.

Since in the case of the patient with chronic nephritis an albumin peak is clearly visible in the electrophoresis graph, it is assumed that the protein remaining in solution at the salt concentration of 268 gm. per liter is for the most part albumin, notwithstanding the lack of a distinct peak in this part of the differentiation diagram.

To calculate the percentage of each of the three serum protein fractions in the four sera under investigation, the quantity of protein, precipitated by Na_2SO_4 solutions, containing 185 and 268 gm. per liter, was estimated from the graphs. In Table II these protein values are compared with the results of the electrophoresis experiments.

DISCUSSION

In recent literature, the opinion has sometimes been held that concentrated salt solutions so seriously denature the serum proteins that most of the data obtained with these methods of investigation are of little value. In this connection a very elaborate fractionation of pig serum, published by Svensson (7), is interesting. In spite of rather violent reprecipitations with ammonium sulfate solutions over a period of 6 weeks, the electrochemical properties of the proteins were not demonstrably altered. Though it cannot be denied that concentrated salt solutions can alter the structure of the proteins, Svensson's experiment proves that these alterations need not be very radical.

Of methods involving treatment with concentrated salt solutions, the determination of solubility curves causes the least denaturation because in this method alone all of the reprecipitations are omitted. The constant locations of the precipitation maxima of albumin and euglobulin in highly pathological human sera lend support to this view.

Concerning the identity of our euglobulin with γ -globulin, and of our pseudoglobulin with $\alpha + \beta$ -globulin, some interesting data have been published recently. Svensson (8) showed that 33 per cent saturation with ammonium sulfate yielded a pure γ -globulin precipitate in horse and swine sera. The filtrates, after 40 per cent saturation in horse and swine sera, 45 per cent in cow serum, and 35 per cent in rabbit serum, were free from γ -globulin. As cow serum contains a large amount of several γ -globulins (average 37.8 per cent), and rabbit serum the smallest amounts found in the sera investigated (average 21.4 per cent) (7), the range of precipitation of this protein must be wider in the first species than in the second. As a

rule, β -globulins are precipitated by less concentrated salt solutions than are α -globulins, but Svensson states that both globulins have a very wide area of precipitation which causes considerable overlapping of these fractions. This is closely in accordance with our inability to demonstrate one or more maxima of precipitation in the solubility curves of pseudoglobulin. The protein precipitating between 40 and 55 per cent of saturation contained much α -globulin, less β -globulin, and a slight amount of γ -globulin in horse and swine sera.¹ Svensson concludes that there is a close parallelism between mobility and solubility. His statement that, nevertheless, it is not a simple matter to isolate the electrochemical components by ammonium sulfate fractionation is wholly confirmed by the form of our differentiation graphs, which suggest considerable overlapping, especially in the pseudoglobulin area.

In Pedersen's monograph (9), too, several statements can be found which lend support to Svensson's thesis of a parallelism between mobility and solubility of the serum proteins. An interesting argument for the close agreement between electrophoresis curves and differentiation diagrams is offered by Wuhrmann and Wunderly (10). Since the work of Gutman *et al.* (11), it has been known that in multiple myeloma serum protein patterns are widely variable. Frequently a very high γ -peak is found; sometimes a large peak with β -mobility is encountered. In our subject W., the high γ -peak could be separated after 210 minutes into two components, the most mobile of which was probably a Bence-Jones protein. Wuhrmann and Wunderly published electrophoresis diagrams and solubility curves in phosphate solutions of the sera of some myeloma patients. Although their solubility curves are not differentiated, it is clear from their figures that a serum with much γ -globulin shows heavy precipitation with very low salt concentrations. In one of the sera with a large amount of β -globulin, much higher salt concentrations were required for precipitation. If this curve had been differentiated, the figure would probably have shown a well defined peak in the pseudoglobulin region.

Our comparison of differentiation diagrams and electrophoresis curves stresses the high degree of correlation between the two methods of analysis. High percentages for γ -globulin are attended by large amounts of euglobulin, and low albumin values are found by both methods. Undoubtedly there is no absolute conformity between the results of the two methods, but this could hardly be expected.

¹ Pedersen's suggestion (9) of the composite nature of the β -globulin might afford an explanation of the occurrence of γ -globulin in the filtrates of precipitations with such concentrated salt solutions. He claims that β -globulin has a molecular weight of 1.9 to 2.6×10^6 and that its molecule is supposed to be composed of lipides, carbohydrates, 1 globulin and 3 albumin molecules. It may well be imagined that this complex will show reversible dissociation in a medium in which most of the albumins and globulins have been removed by salt precipitation.

In electrophoresis, lipides, migrating with the proteins, add to the refraction of light in the U-tube at the moving boundaries, yielding percentages for several fractions higher than the actual concentrations of protein. This is referable to the unequal distribution of lipides (and carbohydrates) among the electrochemical fractions. As Blix, Tiselius, and Svensson (12) showed, the lipide content of normal human albumin is 3.3 per cent, of α -globulin 11.7 per cent, of β -globulin 18.7 per cent, and of γ -globulin 1.4 per cent. Thorn *et al.* (13) determined the increment of the refractive index per gm. of protein N in the serum of a patient with nephritis. They obtained a figure of 2.07×10^{-4} in contrast with 1.16×10^{-4} for pure albumin solutions. This indicates the strong influence of the globulins, carrying many lipides, on light refraction, especially in subjects with nephrosis. Because with nephrosis the very high lipide contents of the serum are not constant, it is quite possible that the refractive indices of the α - and β -globulins will vary widely in different cases. This would make the accurate determination of the absolute values for these fractions almost impossible.

Here, perhaps, the cause can be found of the important difference between the electrophoresis diagram of subject E. with its high α - and β -globulin peaks and the differentiation diagram without any distinct peak in the pseudoglobulin region. Most probably the high α - and β -globulin values are for the most part caused by high concentrations of lipides. The high total cholesterol content of the serum of this patient (750 mg. per cent, determined 24 days before collecting the blood for this investigation) and the distinct blurring of the electrophoresis diagram beyond the β -peak in the photographs substantiate this view. Although, in the differentiation curve, no distinct pseudoglobulin peak can be shown, the absolute pseudoglobulin content of this serum is rather high (19.7 gm. per liter). The percentage value (38.6 per cent) is by far the highest we have found up to the present time. Consequently, some correlation between differentiation and electrophoresis diagrams seems to exist in this exceptional instance too. Undoubtedly more data are required concerning this point.

There is, however, a difference between the results of the two methods that may be of significance. *In all the cases the euglobulin contents are higher than the γ -globulin value, and the albumin contents by differentiation are always lower than those determined from the electrophoresis diagram.* It is very improbable that this difference is caused by precipitation of large amounts of albumin with the euglobulin, as Pedersen (9) has shown that, after precipitation with 31 to 50 per cent saturated ammonium sulfate solutions, the precipitates do not contain albumin (examined in the ultracentrifuge), provided that the serum is diluted ten times or more. In our precipitation experiments the dilution was 20-fold, in the electrophoresis runs, 4- to 5-fold. It is generally held that with higher serum dilutions with

a given salt concentration the protein contents of the filtrates are relatively higher. Because in the precipitation experiments (with the higher dilutions) the lower albumin values are found, the difference of the dilutions cannot be the cause of the discrepancy between the results of electrophoresis and differentiation.

There is far from unanimous agreement about the normal distribution of electrochemical fractions in human serum, the results obtained by the various authors differing widely with buffer composition and protein concentration. Svensson (7) claims that false boundaries are smallest and boundary anomalies are depressed to negligible values when mixtures of phosphates and NaCl, NaF, or LiCl, with a total ionic strength of 0.20 to 0.25, are used as a buffer, while protein concentrations vary between 1.0 and 1.5 per cent. His recent analyses for different animal species, made under "standard conditions," have given decidedly lower albumin and higher γ -globulin contents than earlier observations. The observations of Perlmann and Kaufman (14), too, showed lower albumin values in human plasma when buffer concentrations were raised and protein concentrations lowered. The same holds true for the values for human sera obtained, with Svensson's technique, by Olhagen (15). The averages of his observations are to be found in Table II. His albumin values are lower than those recorded earlier by other authors. Westermann's normal values agree fairly closely with those of Olhagen, notwithstanding somewhat higher protein concentrations (2 per cent, Olhagen: $0.28 \times 6.25 = 1.7$ per cent) and a lower phosphate concentration of the buffer. As the differences between the plus and minus side in Westermann's experiments and in the electrophoresis runs of this investigation are larger than in Olhagen's series, this agreement is rather surprising. Perhaps the δ - and ϵ -boundaries have raised our γ -globulin values, while these were depressed by the less ideal protein concentration and buffer composition. It is possible that this produces almost identical results in normal sera, while in pathological sera with large amounts of γ -globulin the latter factors prevail, resulting in too low values for this protein. So long as the real distribution of electrophoretic components cannot be finally settled, it seems reassuring that recent improvements in this technique result in normal values which approximate those obtained by the differentiation of solubility curves.

It certainly seems justifiable to attach appropriate value to the determination of the three main proteins of serum by single precipitation with sodium sulfate solutions of 185 and 268 gm. per liter. The normal values derived from these and earlier observations (1, 2) are presented in Table IV.

Of some interest is the shift to the left of the maximum of precipitation of the euglobulin in pathological sera. This phenomenon is in excellent agreement with Grönwall's recent observations (16). He found that serum

albumin has an effect upon euglobulin, increasing its solubility. It is conceivable, then, that a shortage of albumin, such as occurred in all our pathological sera, will cause euglobulin to precipitate at lower salt concentrations.

TABLE IV

Normal Values of Protein Fractions by Precipitation with Sodium Sulfate Solutions

Protein fraction	Sodium sulfate	No. of estimations	Protein		
			Maximum	Minimum	Average
	<i>gm. per l</i>		<i>gm per l.</i>	<i>gm. per l.</i>	<i>gm per l.</i>
Euglobulin .	185	2	20.8	20.2	20.5
Pseudoglobulin .	268	9	16.4	10.8	14 0
Albumin		9	47.5	39 5	43.4

SUMMARY

1. Solubility curves of the serum proteins in concentrated sodium sulfate solutions are presented, together with their differentiation diagrams and the electrophoresis curves of the same samples. One normal and three pathological sera are included in this investigation.

2. Concentrations of sodium sulfate equal to 185 and 268 gm. per liter proved best for the determination of the euglobulin, pseudoglobulin, and albumin contents of normal and pathological human sera.

3. Comparison of the percentage distribution of euglobulin, pseudoglobulin, and albumin with γ -, $\alpha + \beta$ -globulins, and albumin of electrophoresis experiments showed a fair degree of correlation between the two methods of analysis.

4. The reasons, probably of a technical nature, why absolute agreement could not be obtained between the results of the two methods are discussed in detail.

The author is indebted to Professor J. G. G. Borst for general supervision of this work, to Dr. H. S. Frenkel, Director of the State Institute for Veterinary Research, for his kind hospitality in this Institute, to Dr. L. W. Janssen for performing the electrophoresis experiments and for valuable criticism, and to the technician, Mrs. W. Hallie-Martens, for the protein determinations necessary for the analysis of solubility curves.

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SERUM PROTEIN FRACTIONATION: A COMPARISON OF SODIUM SULFATE PRECIPITATION AND ELECTROPHORESIS

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Techniques of fractionating serum proteins for clinical use must be simple and convenient. For many years Howe's (1) method has been used almost universally. However, work coming from several laboratories suggests that the procedure can no longer be regarded as satisfactory. Both Butler *et al.* (2) and Luetscher (3) have pointed out discrepancies in the method, and recently Dole (4) has shown its inaccuracy as compared to electrophoresis. In 1945 Pillemer and Hutchinson (5) presented a technique in which methanol precipitation was used, which compared favorably with electrophoretic analysis.

Majoor (6), in 1942, by determining the solubility curves of human sera, concluded that the concentration of 21.5 per cent of sodium sulfate lacked theoretical justification and suggested instead the concentration of 26.8 per cent. He further found that a concentration of 19.6 per cent would separate the globulin into two fractions, which he named pseudoglobulin and euglobulin.

That there is a clinical need for accurate serum protein fractionation has long been evident. Because of the time-consuming nature of electrophoretic analysis, this technique cannot be employed for routine clinical use. It was thought advisable to compare the technique of Majoor with electrophoretic analysis before adopting the former as a clinical procedure. Accordingly, simultaneous fractionations by both methods were carried out on a variety of sera.

Methods

Venous blood, under oil, was obtained in the postabsorptive state with a minimum of stasis. After separation of the serum, total protein was determined in duplicate by the macro-Kjeldahl technique with mercuric oxide as a catalyst. Non-protein nitrogen was determined by the method of Bock and Benedict (7).

The sodium sulfate solutions used for precipitation were prepared as

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follows: 268.6 gm. of anhydrous Na_2SO_4 were dissolved in distilled water, so that the final volume was 1 liter at 37° . For the other solution, 196.3 gm. of anhydrous Na_2SO_4 were dissolved in a similar manner.¹

Precipitation was carried out in the incubator at 37° for either 6 hours or overnight. Filtration was accomplished in the incubator, and, as a precaution against evaporation, the funnels were covered with watch-glasses. Total nitrogen was determined on aliquots of the filtrates, which were then handled in the same manner as for the total proteins. All analyses were carried out in duplicate and frequent blank determinations were made on all reagents.

The non-protein nitrogen was subtracted from the total nitrogen before converting the latter to protein, with the factor 6.25. The calculation of the fractions was made in the following manner.

Filtrate 26 8% Na_2SO_4 precipitation = albumin

Total protein minus albumin = total globulin

Filtrate 19 6 % Na_2SO_4 precipitation minus albumin = pseudoglobulin

Total globulin minus pseudoglobulin = euglobulin

The electrophoretic studies were conducted in the Tiselius apparatus equipped with the Philpot scanning device. The 0.1 N veronal buffer of Longsworth at pH 8.6 was used throughout. Each specimen of serum was diluted with 2 parts of buffer, except one specimen with extremely high globulin content when the dilution was 1:5. The diluted sera were dialyzed for 48 hours at 5° against 2 liters of the buffer used for electrophoresis. The electrophoretic studies were conducted at a bath temperature of $2-3^\circ$ and a potential gradient of 5 to 6 volts per cm. Each analysis was continued until complete separation of the components occurred. Photographs were taken and the magnified projections of the diagrams obtained were traced and examined in the usual manner, the descending boundary being used for the measurement of all the peaks except β -globulin, for which the ascending boundary was employed. α_1 -Globulin and α_2 -globulin were considered to be pseudoglobulins; β - and γ -globulins constituted euglobulin.

One attempt (Subject 16) to extract protein-bound lipide with alcohol and ether was unsuccessful, owing to the inability to reconstitute the serum after extraction. In none of the subjects was there any demonstrable hyperlipemia.

Results

Table I shows a comparison of the values for the various fractions by both methods. The mean differences of ± 0.16 gm. per cent for albumin, ± 0.16 gm. per cent for total globulin, ± 0.15 gm. per cent for pseudoglobulin

¹ 30 cc. of the above solution when added to 1.0 cc. of serum yielded final concentrations of 26 and 19 per cent respectively.

TABLE I
Comparison of Electrophoresis and Salt Fractionation

Serum No	Diagnosis	Total protein		Albumin		Total globulin		Pseudoglobulin		Euglobulin	
		gm. per cent	cm. per cent	Electrophoresis	Na ₂ SO ₄	Electrophoresis	Na ₂ SO ₄	Electrophoresis	Na ₂ SO ₄	Electrophoresis	Na ₂ SO ₄
		gm. per cent	cm. per cent	gm. per cent	cm. per cent	gm. per cent	cm. per cent	gm. per cent	cm. per cent	gm. per cent	cm. per cent
1	Normal	6.90	4.38	4.38	4.38	2.52	2.52	1.06	1.04	1.46	1.48
2	"	7.57	4.50	4.42	4.42	3.07	3.15	1.08	1.17	1.99	1.98
3	"	7.04	4.27	4.51	4.51	2.77	2.53	1.10	1.02	1.67	1.51
4	"	7.02	4.75	4.76	4.76	2.27	2.26	0.92	0.97	1.35	1.29
5	"	7.38	5.00	4.92	4.92	2.38	2.46	0.98	1.05	1.40	1.41
6	"	7.15	4.63	4.49	4.49	2.52	2.66	0.72	0.64	1.80	2.02
7	Multiple myeloma	6.53	4.43	4.05	4.05	2.10	2.48	0.89	1.04	1.21	1.44
8	Cirrhosis of liver	5.93	2.77	2.70	2.70	3.16	3.23	0.67	0.75	2.49	2.48
9	Diabetes mellitus	5.43	2.64	2.82	2.82	2.79	2.61	1.02	0.99	1.77	1.62
10	"	5.94	3.44	3.52	3.52	2.50	2.42	0.69	0.62	1.81	1.80
11	Infected foot	6.82	2.79	2.79	2.79	4.04	4.03	1.79	1.43	2.25	2.60
12	Myocardial infarct	5.16	2.91	3.00	3.00	2.25	2.16	0.93	0.81	1.32	1.35
13	Duodenal ulcer	5.43	2.86	2.75	2.75	2.57	2.68	0.98	0.84	1.59	1.84
14	Carcinoma of liver	6.92	2.30	2.58	2.58	4.62	4.34	1.25	1.08	3.37	3.26
15	" " pancreas	6.06	2.83	3.03	3.03	3.23	3.03	1.26	0.96	1.97	2.07
16	Multiple myeloma	10.93	1.71	1.66	1.66	9.22	9.27	1.07	0.97	8.15	8.30
17	Erythema nodosum	6.71	3.83	3.65	3.65	2.88	3.06	1.03	1.01	1.85	2.05
Difference.		Maximum	0.38	0.38	0.38	0.38	0.38	0.36	0.36	0.35	0.35
		Minimum	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.01	0.01
		Mean	±0.16	±0.16	±0.16	±0.16	±0.16	±0.15	±0.15	±0.15	±0.15

lin, and ± 0.15 gm. per cent for euglobulin are practically identical. The agreement between the two methods is rather remarkable when one considers the range of abnormal sera studied.

Table II lists a series of normal sera which have been fractionated by the Majoor method for use in this laboratory as standard reference values.

TABLE II
Normal Serum Proteins'

		Total protein	Albumin	Total globulin	Pseudo-globulin	Euglobulin
		gm per cent	gm per cent	gm per cent	gm per cent	gm. per cent
Females	1	6.86	4.23	2.63	1.08	1.55
	2	6.79	4.54	2.25	0.96	1.39
	3	6.78	4.69	2.09	0.62	1.47
	4	7.72	4.66	3.06	0.85	2.21
	5	7.01	4.15	2.86	1.36	1.50
	6	7.24	4.77	2.47	0.88	1.59
	7	7.22	4.57	2.65	0.94	1.71
	8	6.37	4.72	1.65	0.72	0.93
	9*	7.57	4.42	3.15	1.17	1.98
Mean.		7.08	4.53	2.58	0.98	1.63
" deviation		± 0.39	± 0.21	± 0.45	± 0.21	± 0.34
Males	10	7.29	4.40	2.89	0.85	2.04
	11	6.56	4.38	2.18	0.89	1.29
	12	7.47	4.63	2.84	1.12	1.72
	13	7.66	5.11	2.55	1.04	1.51
	14*	6.90	4.38	2.52	1.04	1.48
	15*	7.04	4.51	2.53	1.02	1.51
	16*	7.02	4.76	2.26	0.97	1.29
	17*	7.38	4.92	2.46	1.05	1.41
	18*	7.15	4.49	2.66	0.64	2.02
Mean.		7.17	4.63	2.55	0.96	1.61
" deviation....		± 0.31	± 0.24	± 0.22	± 0.14	± 0.27

*Included in Table I

SUMMARY

The values obtained by the separation of the proteins of human sera by a method with 19.6 per cent and 26.8 per cent sodium sulfate have been compared to the electrophoretic analysis of the same sera. The sodium sulfate technique yields three fractions, namely albumin, pseudoglobulin, and euglobulin. The pseudoglobulin corresponds to the sum of the α_1 - and α_2 -globulins, and the euglobulin corresponds to the sum of the β - and γ -globulins, as determined by electrophoresis.

The author is grateful to Dr. John P. Peters for advice and encouragement and to Dr. Abraham White for assistance in the electrophoretic analyses. Miss Mary O'Keefe aided considerably in performing some of the analyses in the normal series.

Addendum—The author has recently had the opportunity to review a paper by Dr. C. L. H. Majoor in which sodium sulfate fractionation was compared with electrophoretic analysis. Fair agreement was found to exist between the two methods in a study of four sera. However, certain discrepancies between his technique and that presented above are apparent. Majoor considered that pseudoglobulin consisted of α - and β -globulins and that euglobulin and γ -globulin were identical. These discrepancies are easily explained by the difference in sodium sulfate concentrations used for the precipitation of euglobulin, 18.5 per cent (Majoor) versus 19.6 per cent (Milne), and by the fact that the author used the veronal buffer at pH 8.6 for his electrophoretic analyses, whereas Majoor employed a phosphate buffer at pH 7.6. The former buffer, we believe, is more satisfactory, since it distinguishes two α -globulin fractions and separates γ -globulin distinctly from any boundary peak at the buffer serum junction.

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THE HYDROLYSIS OF YEAST RIBONUCLEIC ACID BY RIBONUCLEINASE*

I. THE EXTENT OF HYDROLYSIS AND THE PREPARATION OF RIBONUCLEINASE-RESISTANT FRACTIONS AFTER RIBONUCLEINASE TREATMENT

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The isolation of the nucleotides or their well characterized salts after treatment of yeast ribonucleic acid with ribonuclease¹ has shown that the action of the enzyme consists, in part, in the liberation of mononucleotides (1). It has also been suggested from the work of Kunitz (2) and from the small yields of nucleotides isolated that in addition to the formation of the diffusible nucleotides a relatively non-diffusible fraction remains, which unlike the mononucleotides is precipitated in acid solution in the presence of uranium ions. It has not been clear, however, whether the non-diffusible fraction consisted of unchanged nucleic acid or of a resistant fraction not subject to further hydrolysis by the enzyme. Experiments by Zittle (3) favored the former conclusion when it was demonstrated that either guanylic or adenylic acid or the mixture of nucleotides, produced by the action of alkali on yeast nucleic acid, inhibited hydrolysis as measured by the amount of glacial acetic acid-insoluble material produced. In a later publication, however, Zittle has stated that the high polymer fraction obtained from the digestion mixture by precipitation with alcohol was not further hydrolyzed by ribonuclease (4).

In the present paper the question whether or not yeast ribonucleic acid is completely hydrolyzed by ribonuclease has been studied by carrying out the hydrolysis under conditions which allowed the dialysis of the ribonucleotides as they were produced. Measurement of the rate of dialysis of organic phosphorus showed that after a preliminary rapid rate, corresponding to the dialysis of the mononucleotides, a slower relatively constant dialysis rate was reached which was not affected by further addition of enzyme. The results show that a relatively non-diffusible fraction is left which is resistant to further hydrolysis by ribonuclease under these con-

* Taken in part from a thesis submitted by F. H. Carpenter in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Stanford University, 1944.

¹ Kunitz' ribonuclease.

ditions.² This material was characterized by analyses for purine nitrogen, guanine nitrogen, and phosphorus. It differs in composition from the original nucleic acid in containing relatively larger amounts of purine nitrogen, and, when obtained by ribonucleinase treatment of commercial yeast nucleic acid can be fractionated into two parts, one insoluble in 80 per cent acetic acid and another insoluble in equal parts of 80 per cent acetic acid and alcohol.

EXPERIMENTAL

Ribonucleinase—A sample of crystalline ribonucleinase kindly furnished by Dr. M. Kunitz was used. It had been crystallized three times from ammonium sulfate solution and four times from alcohol.

Purified Ammonium Ribonucleate—100 gm. of yeast nucleic acid (Eastman Kodak Company) were dissolved in 200 ml. of water by the addition of sufficient ammonium hydroxide to bring the pH of the solution to about 5.8. The nucleic acid was then precipitated with 5 volumes of glacial acetic acid. After the precipitate had been washed with 50 per cent alcohol and alcohol and ether, 50 gm. of nucleic acid were obtained. It was redissolved with the aid of ammonia, and the solution placed in cellophane bags and dialyzed against 30 liters of distilled water over a period of 24 hours. The dialyzed solution was concentrated *in vacuo* at 40° to a volume of 100 ml., and the ammonium nucleate precipitated by the addition of 8 volumes of 95 per cent alcohol. The yield after thorough washing with alcohol and ether was 20 gm.

Extent of Hydrolysis by Ribonucleinase A preliminary experiment in which a solution of ammonium ribonucleate and ribonucleinase was dialyzed showed that about 66 per cent of the phosphorus had dialyzed after 27 hours. Addition of more ribonucleinase and dialysis for another 24 hours resulted in the loss of an additional 4 per cent of phosphorus. It was evident, therefore, that the rapidly diffusing constituents were largely eliminated under the conditions used after dialysis for less than 27 hours.

In order to find more accurately the per cent of nucleic acid hydrolyzed, a second experiment was performed in which the rate of dialysis of organic phosphorus per hour was determined over the first 7 hour period as well as after 24 hours. 25 ml. of ammonium nucleate solution, containing 25 mg. per ml. at pH 7.7, and 6 mg. of ribonucleinase were dialyzed at room temperature against 18 liters of distilled water over a period of 24 hours. During the first 7 hours 500 ml. of dialysate per hour were collected at hourly intervals, analyzed for organic phosphorus, and the rate of dialysis calculated as per cent of total phosphorus dialyzed per hour. The rate be-

² In a recent paper Carter and Greenstein (5) report that the rate of dialysis of ribonucleic acid is greatly increased in the presence of sodium chloride.

tween 7 and 24 hours was calculated from analysis of the contents of the dialysis bag after the 24 hour period. The rates plotted against time are shown in Fig. 1. It may be seen that the rate decreased rapidly after 5 hours and reached a relatively constant value after about 10 hours. The calculation of the per cent of hydrolysis on the assumption that the nucleotides are completely removed during this time gives a value of about 50 per cent of the original purified sample. A control dialysis experiment on a solution of the purified ammonium nucleate under similar conditions in the

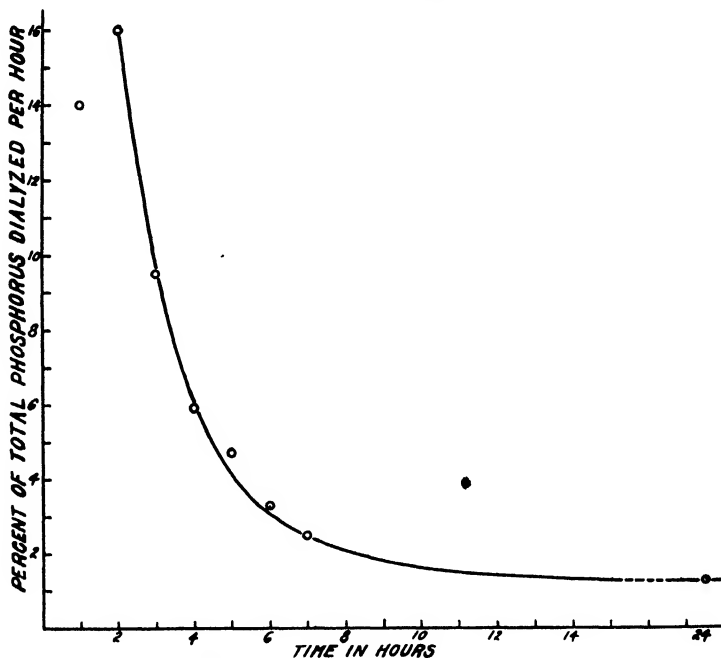


Fig. 1. Rate of dialysis of organic phosphorus liberated from ribonucleic acid by ribonucleinase.

absence of ribonucleinase showed that about 26 per cent of the original nucleic acid had dialyzed after 24 hours.

In order to characterize the fraction resistant to the action of ribonucleinase, the material remaining after dialysis was analyzed for purine nitrogen (6), guanine nitrogen (7), and phosphorus (8). Guanine nitrogen was determined on an aliquot of the acid hydrolysate from the total purine determination after precipitation as the copper-bisulfite complex. The latter was decomposed with hydrogen sulfide and the filtrate analyzed colorimetrically with the phenol reagent (7). The values obtained were then compared with similar analyses carried out on the original nucleic acid solu-

tion of ammonium nucleate and the dialyzed control. The results, expressed as total purine nitrogen, guanine nitrogen, and adenine nitrogen to phosphorus ratios, are given in Table I. The values calculated for a tetranucleotide as given by Levene (9) are also shown. It is evident from the results that the relatively non-dialyzable fraction differs from the original nucleic acid in containing a larger concentration of purine nitrogen and in particular of guanine nitrogen. Of interest is the large discrepancy between the values for guanine to phosphorus and adenine to phosphorus ratios in either the original nucleic acid solution or the dialyzed control as compared to those required by the tetranucleotide theory. While the ratio of purine nitrogen to phosphorus is in agreement with the theory as found also by Graff and Maculla (10), the purine fraction contained approximately twice as much guanine as adenine nitrogen.

TABLE I

Nitrogen Distribution of Ribonuclease-Resistant Fraction and Purified Nucleic Acid Controls

	Purified ribonucleic acid	Purified ribonucleic acid after dialysis	Ribonuclease- resistant fraction	"Statistical" tetranucleotide (Levene)
<u>Total purine nitrogen</u> Phosphorus	1.13	1.07	1.47	1.13
<u>Guanine nitrogen</u> Phosphorus	0.70	0.71	1.03	0.565
<u>Adenine nitrogen</u> Phosphorus	* 0.43	0.35	0.44	0.565
<u>Guanine nitrogen</u> Adenine nitrogen	1.6	2.0	2.3	1

The large amounts of guanine found with the phenol reagent (7) suggested that other constituents of nucleic acid might also give a color reaction under the conditions used. While it is known that adenine does not react, no information was available as to whether or not the pyrimidine nucleotides would give this reaction. It was logical to use the nucleotides rather than the corresponding nucleosides because it is known that the former rather than the latter are produced by mild acid hydrolysis of nucleic acid as carried out in the total purine determination. Approximately 2 mg. each of cytidylic acid and diammonium uridylate (approximately 5 times the maximum quantity of guanine used in the procedure of Hitchings) failed to give any color with the phenol reagent in the presence of sodium carbonate.

Preparation of Ribonuclease-Resistant Fractions—It was of interest to prepare the ribonuclease-resistant fraction in solid form to compare its general properties with the original ribonucleic acid and to determine whether

the isolated material was also resistant to further hydrolysis by ribonucleinase. Approximately 76 gm. of commercial yeast nucleic acid were dissolved in 2 liters of ammonia water at pH 6. The solution was placed in a large cellophane bag equipped with a motor-driven stirrer and dialyzed against distilled water. At the start of the dialysis 76.6 mg. of ribonucleinase were added in four equal portions at 6 hour intervals. Dialysis was continued for a total of 46 hours. Analysis of the solution remaining in the bag for total phosphorus showed that approximately 60 per cent of the original nucleic acid or its split-products had dialyzed during this time. The solution which remained was concentrated *in vacuo* at 40° to about 250 ml., filtered through Celite, and diluted with 7 volumes of glacial acetic acid. A white amorphous precipitate formed which was filtered with suction. The precipitate was washed free of acid by grinding repeatedly in a mortar with 50 per cent alcohol, 95 per cent alcohol and ether, and was air-dried. The product called Fraction A weighed 18 gm. Upon adding an equal volume of 95 per cent alcohol to the acetic acid filtrate, another precipitate separated. This was filtered, washed, and dried by the same procedure used for Fraction A, and weighed 8 gm. (Fraction B).

These fractions resemble closely the original nucleic acid in general properties. They are relatively insoluble in water, forming suspensions that are acid to Congo red paper. Unlike the original nucleic acid, whose particles coalesce under these conditions to form a sticky mass, the particles of the resistant fractions remain relatively dispersed. Fraction A contained 7.8 per cent phosphorus and 12.9 per cent moisture when dried over phosphorus pentoxide at 56°. Fraction B contained 7.9 per cent phosphorus and 12.5 per cent moisture when dried under the same conditions. Preliminary analyses for adenine and guanine indicate that these fractions differ greatly in the amounts of these two constituents present.

Effect of Ribonucleinase on Resistant Fractions—Three stock solutions were prepared by dissolving 0.3 gm. samples of the two resistant fractions and the purified ammonium nucleate in three 50 ml. portions of 0.1 M acetate buffer at pH 5.0. To 4 ml. of each stock solution were added 4 ml. of an enzyme solution containing 0.6 mg. of ribonucleinase per ml. in acetate buffer at pH 4.0. As a control, 4 ml. of acetate buffer at pH 4.0 were added to 4 ml. of each stock solution. All of the solutions were placed in a water bath at 25° for 30 minutes. Then, to each solution were added 8 ml. of a uranium acetate solution, made by dissolving 2.5 gm. of uranyl acetate in 100 ml. of 2.5 per cent trichloroacetic acid, in order to precipitate the unhydrolyzed nucleic acid. After standing for 30 minutes longer in the water bath, the precipitates were filtered off and filtrates were analyzed for phosphorus. The experiment was run in duplicate. The results given in Table II show that in the case of both resistant fractions there is no signifi-

cant difference between the amounts of phosphorus unprecipitated in the enzyme experiment and in the control, whereas in the case of the ammonium nucleate much more of the phosphorus remained unprecipitated in the enzyme-treated sample than in the control. It is evident, therefore, that these resistant fractions, A and B, are not further hydrolyzed by ribonuclease. Of interest is the difference in the solubility of the two fractions in the uranyl reagent. Fraction A which was precipitated by about 87 per cent acetic acid in contrast to Fraction B was also less soluble in the presence of uranium ions and trichloroacetic acid.

TABLE II

Effect of Ribonuclease on Resistant Fractions and on Purified Nucleic Acid As Measured by Organic Phosphorus Formed Soluble in Uranyl Reagent

	Total P concentration	Soluble P					
		After ribonuclease		Control		After ribonuclease*	Control*
		Experiment 1	Experiment 2	Experiment 1	Experiment 2		
	mg. per ml.	mg. per ml.	mg. per ml.	mg. per ml.	mg. per ml.	per cent	per cent
Fraction A	0.113	0.0229	0.0208	0.0218	0.019	19.3	18.1
" B	0.12	0.0279	0.0293	0.0284	0.0294	23.8	24.1
Purified nucleic acid	0.112	0.0876	0.0876	0.0077	0.0089	78.5	7.4

* Average of Experiments 1 and 2.

DISCUSSION

The experiments presented above show that even under conditions in which ribonucleotides are removed as they are formed after treatment of yeast nucleic acid with ribonuclease, about 50 per cent of the original nucleic acid remains as a relatively non-dialyzable fraction which is not subject to further hydrolysis by the enzyme. As this material would be removed from the original commercial nucleic acid by the purification procedures employed, it must be formed as a result of ribonuclease action and must represent a portion of the nucleic acid molecule resistant to further hydrolysis by this enzyme under the conditions used. When commercial nucleic acid was hydrolyzed, the non-dialyzable portion could be further fractionated into at least two parts, one relatively insoluble in 85 per cent acetic acid and another precipitated from the filtrate from the first by an equal volume of alcohol. As no attempt was made to fractionate the resistant fraction from purified nucleic acid by the same procedure, it was not determined whether two fractions are also produced in this case.

The analyses of the original purified nucleic acid for purine nitrogen, gua-

nine nitrogen, and phosphorus show that about twice as much guanine as adenine is present in this preparation. Whereas the phenol reagent as used by Hitchings is not highly specific, guanine is the only compound known to be present in nucleic acid that gives a positive reaction. These results emphasize the fact reported by several workers previously, including Levene (11-14), that all preparations of yeast nucleic acid purified by precipitation with acetic acid do not conform to the "statistical"³ tetranucleotide theory as proposed originally by Levene and as generally accepted by most workers. The reason for the differences is not clear. It is probably related to actual differences in ribonucleic acid samples as well as to differences in methods of hydrolysis and in analytical procedures employed. Differences in the rate of hydrolysis of different yeast nucleic acid samples by alkali have recently been reported by Zittle (4).

The analyses of the enzyme-resistant fraction for purine nitrogen, guanine nitrogen, and phosphorus show a concentration of guanine in this fraction and suggest that ribonucleinase action is concerned to a greater extent with the liberation of pyrimidine than of purine nucleotides.

SUMMARY

Under conditions in which the liberated nucleotides are removed by dialysis, purified yeast ribonucleic acid is converted to a relatively non-dialyzable fraction which is resistant to further action by ribonucleinase. The resistant fraction obtained from commercial nucleic acid could be fractionated into two parts, one precipitated from 85 per cent acetic acid and the other precipitated from the filtrate of the first by an equal volume of alcohol. Analyses of the original purified nucleic acid for purine nitrogen, guanine nitrogen, and phosphorus show about twice as much guanine as adenine, instead of the equimolar ratio required by the "statistical" tetranucleotide theory. The total purine nitrogen to phosphorus ratio was in agreement with the theory. After treatment with ribonucleinase the resistant fraction showed an increase in the amount of guanine nitrogen present.

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³ The term statistical tetranucleotide has been used by Gulland *et al.* (15) to indicate the presence of the four mononucleotides in equimolecular proportion in yeast ribonucleic acid.

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TELLURIUM

IV. EXCRETION AND DISTRIBUTION IN TISSUES STUDIED WITH A RADIOACTIVE ISOTOPE*

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The discovery of some of the effects of tellurium compounds began with the work of Gmelin (1) 123 years ago. The attention of other workers was attracted by the peculiar and unpleasant garlic-like odor that the intake of tellurium and its compounds imparted to the breath. Hansen (2) in 1853 and later Reiser (3), Czapek and Weil (4), Hofmeister (5), and Beyer (6) dealt with different aspects of the same problem.

Interest concerning these compounds has arisen from time to time, with the application as a therapeutic agent to stop the night sweating of tuberculosis patients (Neusser (7), Combemale (8), and others), in the treatment and cure of syphilis (Fournier and Levaditi (9)), and more recently with their increased industrial use.

Most of the work done in the past, concerning distribution and excretion of tellurium, dealt with the administration of rather large amounts, no method being available for the detection and quantitative determination of tellurium in minute quantities. By making use of radioactive tellurium, we have been able to study the fate of this element after administration of much smaller doses (0.1 to 0.5 mg. per kilo of body weight) and to determine the main sites where it is stored and gradually excreted.

Methods and Techniques

Preparation and Separation of Radioactive Tellurium—The radiotellurium used in these investigations was produced at the Massachusetts Institute of Technology cyclotron by the deuteron bombardment of antimony. Two radiotelluriums are apparently formed. One of these is known to be $^{121}_{52}\text{Te}$. According to the recent data of Edwards and Pool (10), it has a half life of 143 days, rather than the 125 days previously reported by Seaborg and coworkers (11). $^{121}_{52}\text{Te}$ results from the nuclear reaction, $\text{Sb}(d, 2n)\text{Te}$. The other radiotellurium, apparently also $^{121}_{52}\text{Te}$ (nuclear

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isomer), is of 17 days half life (10) instead of the 30 day $_{52}\text{Te}^{122}$ or $_{52}\text{Te}^{124}$ reported by Kent and Cork (12).

Following the cyclotron bombardment, the radiotellurium was isolated in the following manner: Concentrated hydrochloric acid, about 3 ml. per gm., and solid potassium chlorate in portions of about 0.5 gm., were added to the bombarded antimony powder until complete solution was achieved. 2 gm. of tartaric acid per gm. of antimony and 20 mg. of carrier tellurium in the form of a sodium tellurite solution were added, and the solution was diluted to attain an approximately 3 N acid concentration. The tellurium was precipitated by adding 10 ml. of 15 per cent hydrazine dihydrochloride to every 100 ml. of solution and then bubbling sulfur dioxide through the boiling solution for 20 minutes. The tellurium precipitate was separated by filtration through a Selas porcelain crucible, dissolved in warm 1:1 nitric acid, and evaporated to dryness. The residue was taken up in 100 ml. of approximately 3 N hydrochloric acid and, to avoid any possibility of contamination with radioactive antimony, 0.8 gm. of antimony trichloride was added. The tellurium was now precipitated once again by the procedure described above. The precipitate was washed first with 3 N hydrochloric acid, then with hot distilled water until chloride-free, and finally with alcohol, and then dried at 105° and weighed.

The elementary tellurium was dissolved in warm 1:1 nitric acid, the solution evaporated to dryness, and just enough sodium hydroxide solution added to the residue to bring it into solution. Then it was diluted with distilled water to the required volume. This was the sodium tellurite solution used throughout the animal experimentation. Two such preparations were made. In one instance 21 mg. of tellurium carrier were added to the bombarded antimony, and approximately 100 microcuries of radiotellurium were obtained. The other preparation contained about 1 millicurie in 18.6 mg. of elementary tellurium.

Detection of Radiotellurium— $_{52}\text{Te}^{121}$ ($t_{1/2} = 143$ days) is converted by means of an isomeric transition into $_{52}\text{Te}^{121}$ ($t_{1/2} = 17$ days) with the emission of a 0.22 m.e.v. γ -ray (10). The 17 day tellurium decays into $_{51}\text{Sb}^{121}$ (stable) apparently by K capture plus emission of a 0.61 m.e.v. γ -ray (10). The indications are, however, that there is also a significant amount of electron emission, due to the high probability of internal conversion of the γ -rays which are emitted by both radiotelluriums. However, in spite of this complex decay scheme, it was still possible to obtain radioactive analyses to within 5 per cent through the detection of these converted electrons by following a procedure similar to that developed by Henriques and coworkers (13) for the detection of S^{35} .

The radioactive determinations were made on elementary tellurium.

Radiotellurium present in the +6 state of oxidation in dilute hydrochloric acid was quantitatively reduced to the element, following the addition of sufficient non-radioactive (carrier) tellurium to produce 4 mg. of precipitate.

The Carius method was used to oxidize the radiotellurium to tellurate. With the tissue samples, this procedure is standard (14). In the case of the respiratory samples the Carius method had to be modified in order (1) to insure no loss of the volatile tellurium compounds during handling, and (2) to remove, owing to the possibility of an explosion during the high temperature oxidation, the large quantity of carbon dioxide condensed in the tube by the liquid air during the collection of the breath sample. This was accomplished as follows: The Carius tube was disconnected from the respiratory apparatus, and after the addition of 1 ml. of fuming nitric acid, it was sealed off. During these manipulations the tube was kept continuously in a Dewar flask containing liquid air. The Dewar flask was now removed and the tube remained at room temperature for at least 1 hour to allow the nitric acid to oxidize the tellurium compounds. The tube was reimmersed in liquid air, opened, and the cooling agent removed to allow the carbon dioxide to escape. It was then resealed and submitted to the standard Carius oxidation.

After oxidation by the Carius method, the solution was washed into a 30 ml. beaker and evaporated to dryness on a hot-plate following the addition of 2 ml. of tellurite carrier (2 mg. of tellurium per ml.). The residue was then dissolved, washed into a 250 ml. Phillips conical flask, and made up to a volume of about 50 ml. with 3 N hydrochloric acid.

2 N permanganate was added dropwise until the solution had a slight purple tinge. Then it was heated until it became colorless once more. Now the reducing agents, 3 ml. of a 15 per cent solution of hydrazine dihydrochloride followed by 5 ml. of a 10 per cent stannous chloride solution, were added. The mixture was boiled vigorously until clearing of the supernatant signified that the precipitation was complete.

The solution was filtered hot and washed alternately with hot water and absolute ethyl alcohol. The elementary tellurium was collected on a 2.15 sq. cm. area of Munktell No. OK filter paper, mounted on a brass disk, and the radioactivity measured inside a Lauritsen electroscope. The details of these steps are essentially identical to those used for benzidine sulfate in the S^{35} work (13).

The purpose of adding the carrier tellurium as tellurite and the subsequent oxidation by permanganate to tellurate was to insure that the oxidation states of both the inert and radioactive tellurium were identical prior to reduction. The specific quantities of carrier tellurium and the two reducing agents which were used, together with the vigorous boiling in the

Phillips conical flask, were quite critical in obtaining a precipitate whose particle size was sufficiently uniform and coarse to result in both the quantitative collection and the uniform distribution on the filter paper.

The accuracy of this analytical procedure was evaluated by making determinations on known volumetric aliquots of radiotellurium in a manner similar to that previously described (13). These data showed that radioassays to 5 per cent could be made on all samples which contained an activity somewhat greater than 5 times the electroscope background (about 6 divisions per hour). An accuracy of approximately 15 per cent was obtained with samples whose activities were of the order of the background.

The sensitivity of the method depends primarily upon three factors: the millicurie strength of the radiotellurium, the amount of carrier tellurium used to isolate the activity, and the detection instrument. The radioactive preparation which contained about 1 millicurie of activity in 18.6 mg. of tellurium metal made possible the determination (to within 15 per cent) of 0.001 and 0.005 γ , respectively, at the beginning and termination of the investigations to be described. With the preparation which contained about 100 microcuries in 21 mg. of tellurium, this sensitivity was about 10 times lower. The substitution of a thin window β -ray counter (13) and the requisite auxiliary electronic counting apparatus would have increased this sensitivity approximately 2.5 times.

Sampling Techniques—We have used rabbits under light urethan anesthesia for the study of respiratory excretion of tellurium, probably as $\text{Te}(\text{CH}_3)_2$.¹ A mask, similar to the one used for metabolism determination in dogs, but of a proportionally smaller size and with two Krogh valves, one serving as inlet and the other as outlet, was used.

The expired air went through a Carius tube, immersed in liquid air, which completely trapped the odorous volatile compound, and the determination of tellurium was carried out according to the technique described before.

Samples of tissues and organs were obtained immediately after killing the animal with a blow on the head (rabbits and rats) or by injection of nembutal (dogs). The organs and tissues were freed from adhering blood and blood clots, but no attempt was made to perfuse them to eliminate the blood present within the tissue.

Blood was obtained by heart puncture in rabbits and rats, and by venipuncture in dogs. Clotting was prevented by the addition of heparin. In the rabbit experiments, bile and urine were taken from the bladder. To study excretion, female dogs were kept in metabolism cages and

¹ There is no direct proof that this is the volatile compound present in expired air (Schultz and Lewis (15) and Challenger (16)).

catheterized for urine at the end of each period, the urine being added to that collected from the cage.

Excretion of Tellurium in Expired Air in Rabbits—It was necessary to put the rabbits under light urethan anesthesia in order to be able to collect the volatile compound for periods of 6 to 8 hours. The anesthesia was produced by intraperitoneal injection of 4 ml. per kilo of a 25 per cent urethan solution, repeated if necessary. Urethan did not interfere with the formation of the volatile compound. The tellurite solution, containing radiotellurium, was injected into the marginal vein of the ear. The garlic breath appeared 2 or 3 minutes after injection of the tellurite. A small dose of sodium tellurite, as little as 50 γ of tellurium per kilo and probably less, was sufficient to produce the garlic breath.

In spite of being able to produce a strong garlic breath with 100 and 300 γ per kilo, we could not detect the tellurium present in the expired air after a 2 hour collection period. This gives an idea of the strength of the odor, since with our method of determination we were able to detect as small an amount as 0.01 γ of tellurium. We had to increase the amount injected to 500 γ per kilo of body weight in order to be able to detect the tellurium in expired air. This has been the dose used in all our experiments on rabbits. In four of the experiments, 20 mg. per kilo of body weight of a freshly prepared solution of ascorbic acid (Merck, U. S. P.) in distilled water were injected intraperitoneally 20 to 30 minutes before the tellurite injection.

The results obtained under these conditions are shown in Table I. The garlic breath had disappeared 24 hours after the injection of tellurite. If we consider the rate of excretion to be constant during these 24 hours, and assume a maximum excretion of 0.08 γ over a period of 3 hours (Rabbit 12), the total amount excreted through the lungs would have been 0.64 γ . This represents less than one-thousandth of 1100 γ , which was the total amount injected into this rabbit. This shows that the respiratory tract is not an important channel of excretion under the conditions of our experiments.

Though no definite conclusion can be drawn concerning the effect of ascorbic acid, observations made in workers with garlic breath² show a definite decrease or even disappearance of it upon ascorbic acid administration.

One of the few earlier attempts to detect the presence of tellurium in expired air after administration of tellurium compounds was made by Hofmeister (5) and is of a qualitative type. The presence of selenium in expired air after administration of selenium compounds has been studied by Schultz and Lewis (15), who administered ordinary sodium selenite, and by McConnell (17), who administered radioactive selenate to white rats.

² DeMeio, R. H., to be published.

The important fact is that in our case, in which the rabbit and intravenous injection were employed, the total amount excreted through the respiratory tract is less than 0.1 per cent of the total injected, whereas Schultz and Lewis (15), using the subcutaneous route in white rats, found an excretion of selenium of from 17 to 52 per cent in 8 hours, and McConnell (17) found 3 to 10 per cent in 24 hours. This difference may be related to the greater speed of elimination in the rat than in the rabbit, as can be seen from our

TABLE I

Tellurium in Expired Air after Intravenous Administration of $\text{Na}_2\text{Te}^\text{O}_3$ to Rabbits*
Dose, 500 γ of Te per kilo of body weight under light urethan anesthesia.

Rabbit No.	Weight	Collection period	Te in expired air	Observations
	kg		γ	
5	2 19	2 hrs., 30 min.	0.045	
6	1.9	2 " 30 "	0.039	
7	2.3	1st 3 hrs.	0.036	
		2nd 3 "	0 04	
		3rd 1½ hrs	0 014	
8	1.86	1st 3 hrs	0.016	
		2nd 3 "	Lost	
		3rd 1½ hrs	0 008	
12	2 2	1st 3 hrs	0.075	
		2nd 3 "	0.046	
14	1 84	1st 3 "	0.014	
		2nd 3 "	0.012	
9	1.87	1st 3 "	0 012	20 mg. per kilo ascorbic acid intraperitoneally
		2nd 3 "	0.013	
10	2.0	1st 3 "	0.066	" "
		2nd 2 "	0 015	
11	2.19	1st 3 "	0.017	" "
		2nd 3 "	0.012	
13	2.0	1st 3 "	0 064	" "
		2nd 3 "	0.063	

results on tellurium distribution, and to a greater rate of elimination for selenium.

Distribution in Rabbit Tissues and Body Fluids—Six of the rabbits used for the study of tellurium in expired air were killed by a blow on the head and samples of various tissues, blood by heart puncture, and bile and urine from the bladder, were obtained for analysis. Samples of skeletal muscle and bone were obtained from the hind legs. Tellurium determinations were carried out by the method already described.

Table II shows the results obtained and the distribution pattern. The most conspicuous and outstanding fact is the great concentration of tellurium in the kidneys, which amounts to 3 to 10 times the concentra-

TABLE II

Distribution of Te in Rabbit Tissues and Body Fluids after Intravenous Administration of $\text{Na}_2\text{Te}^\text{O}_3$*

Dose, 500 γ of Te per kilo of body weight under light urethan anesthesia; 20 mg. per kilo of ascorbic acid intraperitoneally to Rabbits 10, 11, 9, and 13, 20 to 30 minutes before tellurite injection.

The results are expressed as micrograms of Te per 100 gm. or ml.; the figures in parentheses represent the total Te in micrograms.

Rabbit No.	10	11	9	13	12	14
Weight, kg.	2	2.19	1.87	2	2.2	1.84
Total Te given, γ	1000	1095	935	1000	1100	920
Killed after, hrs.	5½†	10½‡	23½	24	24½	22
Bone	31.2	137		111	39.7	106
“ and marrow	83.7					
“ marrow	90.3	69		55	76.4	39.8
Encephalic matter	13.8 (1.41)	5.7 (0.52)	6.1 (0.5)	7.1 (0.61)	5.9 (0.62)	9.0 (0.83)
Heart			188.5 (6.9)	211 (9.3)	192 (9.6)	24 (0.89)
Intestine, small			39.2	42.8	37.3 (11.9)	29.4
“ large			84.9	57.0	68.4 (31.9)	37.6
Kidney	685.9 (107.3)		660.1 (75.3)	876 (115)	575 (120)	1400 (154)
Liver	111.7 (81)		103.3 (73.9)	71 (53.3)	83 (59)	89 (59.5)
Lungs	196.1 (21.8)		171 (10.9)	160 (12.6)	176 (18)	134 (8.7)
Muscle	13.6	1.06	10	8.3	9.6	8.6
Spleen			171 (2.3)	174 (2.51)	289 (4.38)	118 (2.1)
Stomach			32.4 (9.8)	41 (8.46)	71 (18)	49 (8.1)
Teeth		9.8		22.7		22.3
Testes				21.1 (0.17)	63 (0.49)	
Bile...			161.2	224	36	162
Blood			22.5	22.9	34.3	19.5
Urine				174	310	

† Died.

‡ Found dead.

tion in the next richest organ. Similar results, as far as the kidneys are concerned, were obtained by Levaditi and Manin (18) by injecting or feeding elementary tellurium and sodium tellurite to rabbits.

Some radioautographs, obtained from rat kidneys, show an uneven distribution of tellurium, with a larger concentration in the cortex.³

The high concentration in the kidneys is undoubtedly due in part to the fact that tellurium is being concentrated for excretion by this organ, particularly in our case in which it was given in a water-soluble form, sodium tellurite.

In most instances heart, lung, and spleen show approximately the same concentration of tellurium, which is about 1.5 to 2 times as great as that in the liver. In order of decreasing concentrations, and lower than the liver, we have bone, bone marrow, large intestine, stomach, small intestine, testes, teeth, skeletal muscle, and encephalic matter.

It is interesting to note the tremendous difference between skeletal muscle and heart muscle, the latter having a concentration of tellurium 20 times larger than the former. Beyer's (6) observation, that stimulated skeletal muscle reduces tellurium compounds more rapidly than the muscle at rest, may explain to a certain extent this difference, since the heart muscle is in activity, while the skeletal muscle is at rest.

The lowest concentration of tellurium is found in encephalic matter. Is this pattern of distribution due to differences in permeability to the tellurite ion, or to the uneven distribution of the mechanisms that act in reducing the tellurite to tellurium and depositing it in tissues, or to both? This question remains to be answered.

In most cases, the tellurium concentration in urine and bile is roughly 10 times as great as the blood concentration, showing that these are two important channels of excretion, urine being, of course, the more important because of the larger volume excreted. As a consequence, the tellurium that has been found in feces after parenteral administration (Mead and Gies (19) and Levaditi and Manin (18)) has at least partially been excreted with the bile.

The administration of ascorbic acid 20 to 30 minutes before the tellurite injection does not seem to modify the distribution pattern.

Our findings concerning encephalic matter do not agree with Jahnel, Page, and Müller's (20) results. They have found 1 mg. of tellurium in 2.3 gm. of brain cortex after intramuscular injection of an oil suspension of elementary tellurium. The difference may be due to a different dosage and length of treatment, which they do not state, and the form in which the tellurium was given.

Similar results concerning distribution in the body were obtained by Rosenfeld and Beath (21) feeding selenium to sheep (see also Moxon and Rhian (22)).

³ DeMeio, R. II., and Jetter, W. W., to be published.

Distribution in Rat Tissues and Body Fluids—Rats were injected intraperitoneally with different doses of tellurium as sodium tellurite, containing the radioactive isotope, and no anesthesia was used. Blood, by heart puncture, and samples of organs were obtained after the animal was killed with a blow on the head. Skeletal muscle and bone were obtained from the hind legs. Table III shows the results obtained, and the distribution pattern is about the same as that observed in rabbits. The important

TABLE III

Distribution of Te in Rat Tissues and Body Fluids after Intraperitoneal Administration of $\text{Na}_2\text{Te}^\text{O}_3$*

The results are expressed as micrograms of Te per 100 gm or ml.; the figures in parentheses represent the total Te in micrograms.

	Rat 1	Rat 2	Rat 3	Rat 4
Weight, gm.	300	235	198	220
Te per kilo, γ	1400	280	300	300
Total Te given, γ	420	66	60	66
Killed after, hrs.	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	24 $\frac{1}{2}$
Adipose tissue			26.5	0
Bone ...		32.5	28.5	1.04
Encephalic matter	21 (0.29)	3.25 (0.04)	11 (1.72)	0
Heart	202 (2.25)	24.7 (0.19)	55 (0.34)	2.16 (0.014)
Intestine, small			26.6	4.8
" large		47.8		
Kidney	1340 (33.2)	143.5 (2.72)	270 (3.84)	16.5 (0.22)
Liver	236 (24.7)	44.5 (4.18)	62.3 (4.24)	3.8 (0.22)
Lungs	248 (2.88)	38.4 (0.43)	59.2 (0.52)	1.03 (0.01)
Muscle	36	9	9.1	1.47 (0.02)
Skin			19.1	0
Spleen	281 (2.98)	36.6 (0.45)	60 (0.47)	2.86 (0.02)
Stomach		29.1 (0.32)	46.1 (0.38)	9.04 (0.09)
Testes				0.39 (0.005)
Tongue			264 (0.87)	0
Blood	428	36.4	15	

difference is that the tissue concentration seems to drop considerably after 24 hours, which is not the case in rabbits. In Rat 3, in which the tongue was analyzed $\frac{1}{2}$ hour after injection, a surprisingly high concentration of tellurium was found. As the tongue was not washed to free it from contaminating saliva, some of the tellurium present may have come from it. That tellurium is probably excreted in the saliva is indicated in many individuals by the presence of a metallic taste after administration of sodium tellurite.

Excretion in Dogs—Because of the ease of catheterization, female dogs were chosen. They received, either by intravenous injection or by oral administration, a solution of sodium tellurite containing radioactive

TABLE IV

Renal Excretion and Blood Concentration of Tellurium after Intravenous Administration of 100 γ of Te Per Kilo of Body Weight, As $\text{Na}_2\text{Te}^\text{O}_3$, to Female Dogs*

Dog No.	Weight	Collection period	Urine volume	Urinary Te	Te excreted per hr.	Total Te excreted	Whole blood Te	Plasma Te	Red blood cell Te†	Hematocrit
	kg.	hrs.	ml.	γ per cent	γ	γ	γ per cent	γ per cent	γ per cent	per cent
0	5.4	0- 1	25	302	75.5	75.5	20.6			
		1- 4	30	28	2.8	8.4	12			
		4- 7	22	22.8	1.7	5	8.2			
		7- 24	238	4	0.56	9.5	5			
1	5.5	0- 1	28	210	58.8	58.8	9.5	4.7	16.8	39.6
		1- 4	33	14.8	1.6	4.9	7.3			
		4- 7	40	7.8	1.03	3.1				
		7- 24	385	2.5	0.57	9.6	4.4			
		24- 48	530	0.86	0.19	4.6				
		48- 72	530	0.85	0.19	4.5				
		72- 96	320	0.58	0.08	1.9				
		96-120	505	0.23	0.05	1.2				
2	3.2	0- 1	5	818	41	41	6.8	7.1	6.4	44
		1- 4	8	64.2	1.7	5.1	4.4			
		4- 7	14	22.3	1.03	3.1	5.6			
		7- 24	164	4.03	0.39	6.6	2.2	3.4	0.84	46.4
		24- 48	96	3.36	0.13	3.23				
		48- 72	100	1.02	0.04	1.02				
		72- 96	74	1.58	0.05	1.17				
		96-120	95	0.008	0.03	0.78				
3	8.8	0- 1	10	1440	144	144	6.7	9.9	3.4	48.5
		1- 4	26	79.3	6.9	20.6	5.5			
		4- 7	22	35.3	2.9	7.8	5.7			
		7- 24	310	4.5	0.82	13.9	3.02			
		24- 48	455	1.8	0.34	8.2				
		48- 72	305	2.02	0.26	6.2				
		72- 96	255	1.47	0.16	3.75				
		96-144	490	0.22	0.023	1.08				

† Calculated.

tellurium at a level corresponding to 100 γ of tellurium per kilo of body weight. Garlic breath appeared in 1½ to 2 minutes after intravenous administration and 20 minutes after oral administration, and was still present after 72 hours, but absent after 100 hours.

The urine was collected for the periods indicated in Tables IV and V, and blood samples (clotting being prevented with heparin) were obtained by venipuncture at the end of the period. The dogs were kept in metabolism cages, and the urine collected was added to that obtained by catheterization at the end of each period.

Tellurium was determined in whole blood and plasma, and the red cell concentration calculated from the hematocrit values. Hematocrit values were obtained by the method described by Wintrobe (23).

The results obtained are presented in Tables IV and V. Renal excretion follows an entirely different pattern when tellurium is administered intravenously than when it is given by stomach tube (see Fig. 1).

TABLE V

Renal Excretion and Blood Concentration of Tellurium after Oral Administration of 100 γ of Te Per Kilo of Body Weight, As $\text{Na}_2\text{Te}^\text{O}_3$, to a Female Dog Dog 4; 8.6 kilos*

Collection period	Volume of urine	Urinary Te	Te excreted per hr.	Total Te excreted	Whole blood Te	Plasma Te	Red blood cell Te†	Hematocrit
hrs.	ml.	γ per cent	γ	γ	γ per cent	γ per cent	γ per cent	per cent
0- 1	16	0.58	0.09	0.09	0.59	0.54	0.65	44
1- 4	28	5.54	0.52	1.55	2.32			
4- 7	24	8.07	0.65	1.94	1.63	2.53	0.51	44.7
7- 24	168	12.5	1.24	21.0	0.84			
24- 48	215	0.75	0.07	1.6				
48- 72	230	0.27	0.03	0.62				
72- 96	200	0.36	0.03	0.71				
96-144	445	0.15	0.014	0.67				

† Calculated.

Let us first examine the results of intravenous administration (Table IV). The bulk is excreted within the 1st hour after injection, excretion slowing down and being particularly low after the first 24 hours (see Fig. 1). From 10.7 to 16.4 per cent of the amount injected (Table VI) is excreted in the 1st hour, reaching a value of 23.4 per cent after 144 hours (Dog 3). A maximum of about 20 per cent of the tellurium injected is excreted through the kidneys in a 5 or 6 day period.

As the intestinal excretion of tellurium is lower than the renal excretion,⁴ and even if considered equal, over 60 per cent will still be left to be accounted for. From our experiments on rabbits we know that the amount eliminated through the respiratory tract is negligible and will probably not amount to more than 1 per cent. This means that more than 60 per cent of the injected tellurium remains in the body after 5 or 6 days. It is easy

⁴ Hamilton, J. G., personal communication.

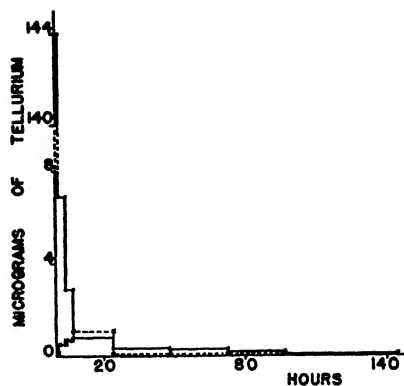


FIG. 1. Renal excretion of tellurium after administration of 100 γ of tellurium per kilo of body weight (as radioactive sodium tellurite) to female dogs. The solid line represents intravenous administration; the dash line, oral administration.

TABLE VI

Percentage of Tellurium Excreted after Intravenous and Oral Administration of 100 γ of Te Per Kilo of Body Weight, As $\text{Na}_2\text{Te}^\text{O}_3$, to Female Dogs*

Dog. No.	Collection period	Total Te given	Total Te excreted	Per cent excreted
	hrs.	γ	γ	
0	1	540	75.5	14.0
	24		98.4	18.2
1	1	550	58.8	10.7
	24		76.4	13.9
	48		81.0	14.7
	72		85.5	15.5
	96		87.4	15.9
	120		88.6	16.1
2	1	320	41.0	12.8
	24		55.8	17.5
	48		59.0	18.5
	72		60.0	18.7
	96		61.2	19.1
	120		62.0	19.3
3	1	880	144	16.4
	24		186.3	21.2
	48		194.5	22.1
	72		200.7	22.8
	96		204.5	23.3
	144		205.6	23.4
4 (Oral administration)	1	860	0.09	0.01
	24		24.58	2.86
	48		26.18	3.04
	72		26.8	3.12
	96		27.51	3.2
	144		28.18	3.28

to realize that the complete elimination of the tellurium left in the body will take several months, if we consider the very low rate of renal excretion established after 72 hours. That tellurium was still present in tissues a long time after injection is shown by our results. We analyzed the kidneys, the organ with the highest concentration, and found 42.5 γ of tellurium per 100 gm. and a total of 21.8 γ in Dog 3 after 11 days of administration, though garlic breath had disappeared 6 days before.

If we compare urine and blood, using the data of the 1st hour, we see that urine has a concentration of tellurium 15 to 215 times as great as blood.

Evidently the readily water-soluble tellurite is rapidly excreted, but as time goes on, tellurium is deposited in tissues and excretion slows down considerably. This behavior indicates that repeated administration of tellurium will lead to an accumulation in the body.

Oral administration produces an entirely different picture (Table V). Though we performed only one experiment, the results seem clear cut. Renal excretion is very low in the 1st hour, increases to reach a maximum between the 7th and the 24th hours, and then decreases again. The total amount excreted after 144 hours represents only 3.28 per cent of the total amount administered by stomach tube. The blood concentration varies somewhat in a similar manner to the urine concentration. This is probably determined by the rate of intestinal absorption and related to it.

The very low percentage of excretion is probably due to the fact that a rather low proportion of tellurium is absorbed in the intestine. Gmelin (1) had already observed a heavy deposition of tellurium in the gastrointestinal tract. Mead and Gies (19) state that the greatest part of ingested tellurium is eliminated with the feces, and when quantities carried into the stomach are small, only traces of tellurium appear in the urine and frequently none can be detected. According to Hamilton⁴ only 25 per cent of the tellurium ingested is absorbed. In agreement with these observations, we found that the kidneys, after 11 days of ingestion, had a tellurium concentration of 9.75 γ in 100 gm. with a total of 4.8 γ , which is about one-fourth of that found in Dog 3 (intravenous administration).

The study of the distribution of tellurium between red blood cells and plasma shows in most instances a higher concentration in plasma than in red cells. This picture may change if the animal should receive tellurium over a long period of time.

SUMMARY

A method for the determination of radioactive tellurium is described. The following observations were made in rabbits which had been injected intravenously with 500 γ of tellurium, as radioactive sodium tellurite, per kilo of body weight. The amount of tellurium excreted through the

respiratory tract varied between 0.012 and 0.08 γ for a 3 hour period. The amount of tellurium excreted in 24 hours thus was less than one-thousandth of the total amount injected.

The largest concentration of tellurium was found in the kidneys. Heart, lung, and spleen showed approximately the same concentrations, which were one-third to one-tenth that in the kidneys, but 1.5 to 2 times greater than in the liver. A still lower concentration was found in skeletal muscle, while the smallest amount was present in encephalic matter. The concentrations shown by other organs and tissues ranged between those of muscle and liver. The concentration in bile and urine was 10 times that found in blood. A similar pattern of distribution was found in rats, with the difference of a sharp decrease in concentration after 24 hours of injection.

The renal excretion in female dogs, after intravenous administration of 100 γ of tellurium (as radioactive sodium tellurite) per kilo of body weight, is high the 1st hour, decreasing sharply afterwards. The total amount excreted in 5 or 6 days represents roughly 20 per cent of the amount injected. After oral administration, the maximum renal excretion is reached between the 7th and the 24th hours, and the total after 6 days represents only 3.28 per cent of the amount administered. After 11 days, tellurium was still present in the kidneys, the concentration being 4 times greater in a dog injected intravenously compared to that in an animal treated orally. Tellurium was present in a higher concentration in plasma than in red blood cells.

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THE EFFECT OF THE HYPOPHYSEAL GROWTH HORMONE ON THE ALKALINE PHOSPHATASE OF RAT PLASMA*

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Decrease in the alkaline phosphatase level in rat plasma following the removal of the pituitary or the administration of adrenocorticotrophic hormone (1) indicates that the hypophysis may play an important rôle in the hormonal control of phosphatase activity. The results reported herein were obtained from a continuation of this study with the growth hormone.

Male rats hypophysectomized at 40 days of age were employed. The intraperitoneal administration of the hormone began either immediately after the operation or 14 days later. The growth hormone was isolated from ox anterior pituitaries by the method previously described (2). The animals were anesthetized with sodium amytal and blood was taken from the inferior vena cava with 4 per cent sodium citrate solution as the anticoagulative agent. The alkaline phosphatase activity was estimated by the procedure described by Binkley *et al.* (3); at least three determinations were carried out on each plasma sample. The Coleman spectrophotometer was employed for recording the color intensity. The plasma nitrogen was determined by the micro-Kjeldahl method. A unit of alkaline phosphatase is defined as that amount of activity which will liberate 1 mg. of phenol in 30 minutes in barbiturate buffer of pH 9.7.

Table I summarizes the experimental results obtained in hypophysectomized male rats. It is evident that the growth hormone caused marked increases in the concentration of the alkaline phosphatase of plasma, whether the hormone was injected immediately after hypophysectomy or 14 days later. When injections began on the day of the operation, the lowest dose (0.01 mg. daily) produced no changes in the enzyme concentration, but dosages of 0.03 to 0.20 mg. caused significant increases in the plasma alkaline phosphatase. There is no correlation between the growth-promoting activity and the increment in the enzyme level, though the highest dose (0.2 mg. daily) was found to be the most effective in increasing the enzyme content in the plasma. The alkaline phosphatase content changes from 3.46 to 6.40 units per 100 mg. of plasma nitrogen; the latter value is significantly higher than that found in normal male rats (1).

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Similar results were obtained when the hormone was administered 14 days after hypophysectomy.¹

In order to ascertain that the effect observed by growth hormone is not due to non-specific action, we have carried out the same experiments in which lactogenic hormone was employed in a daily dose of 0.2 mg. As shown in Table II, there is no difference in the alkaline phosphatase of plasma content between the lactogenic hormone-treated and control animals. It may therefore be said that elevation in the enzyme level in the

TABLE I
Effect of Growth Hormone on Plasma Alkaline Phosphatase of Hypophysectomized Male Rats

Daily dose*	No. of rats	Body weight			Plasma	
		Initial	Autopsy	Gain	Nitrogen	Alkaline phosphatase
Injections begun on day of operation†						
mg.		gm.	gm	gm.	mg. per cc.	units per 100 mg. N
0.00	17	147.1 ± 1.98‡	124.6 ± 2.02	-22.5	8.67 ± 0.16	3.46 ± 0.22
0.01	4	152.0 ± 3.9	136.2 ± 8.3	-15.8	9.00 ± 0.31	3.14 ± 0.12
0.03	10	123.3 ± 3.9	127.4 ± 4.0	4.1	9.18 ± 0.29	4.22 ± 0.26
0.10	12	145.9 ± 3.7	155.1 ± 3.9	9.2	8.60 ± 0.14	4.80 ± 0.39
0.20	10	141.5 ± 4.0	168.6 ± 3.6	27.1	8.89 ± 0.25	6.40 ± 0.85
Injections begun 14 days after operation†						
0.00	9	122.1 ± 3.0‡	112.2 ± 3.2	-2.9	9.71 ± 0.22	3.11 ± 0.34
0.05	10	118.8 ± 2.6	139.4 ± 4.0	21.6	8.63 ± 0.11	5.39 ± 0.64
0.10	9	125.4 ± 4.1	147.1 ± 6.1	21.7	9.35 ± 0.22	4.50 ± 0.36
0.20	9	118.5 ± 2.7	151.2 ± 4.6	33.6	8.70 ± 0.24	6.02 ± 0.98

* Intraperitoneal injections once daily for 15 days except Sunday (thirteen injections).

† Rats operated on at 40 days of age.

‡ Mean ± standard deviation.

plasma caused by growth hormone is specifically due to the characteristic function of the hormone. This effect of the growth hormone can also be demonstrated in non-hypophysectomized animals. Normal female rats of 89 days old were injected² intraperitoneally with 0.5 mg. of hormone

¹ It is of interest to note that the growth-promoting activity of the hormone is higher if the injections began on the day of hypophysectomy instead of 14 days later. From Table I, it may be seen that 0.2 mg. dose produced a weight gain of 49.6 gm. in 15 days when the hormone was administered on the day of operation, whereas the same dose gained 30.7 gm. if injections were begun 14 days after hypophysectomy.

² In these experiments, the animals were fasted 24 hours before autopsy, and heparin was used as the anticoagulative agent instead of sodium citrate.

daily for 20 days. Table III shows that the concentration of alkaline phosphatase in the plasma of injected animals is almost double that in the controls and that the injected group showed weight gains averaging 83 gm. in 19 days, while the controls gained only 17 gm.

TABLE II
Effect of Lactogenic Hormone on Plasma Alkaline Phosphatase of Hypophysectomized Male Rats

Experiment	No. of rats	Body weight			Plasma	
		Initial	Autopsy	Difference	Nitrogen	Alkaline phosphatase
		gm.	gm.	gm.	mg. per cc.	units per 100 mg. N
Control*	17	147.1 \pm 1.98†	124.6 \pm 2.02	-22.5	8.67 \pm 0.16	3.46 \pm 0.22
Injected†	12	140.5 \pm 3.20	122.9 \pm 3.47	-17.6	9.82 \pm 0.26	3.00 \pm 0.30

* Rats operated on at 40 days of age.

† Intraperitoneal injections were begun on the day of hypophysectomy with 0.20 mg. of hormone daily for 15 days except Sunday (thirteen injections).

‡ Mean \pm standard deviation

TABLE III
Effect of Growth Hormone on Plasma Alkaline Phosphatase of Normal Female Rats
Six animals* in each experiment; the female rats were 89 days of age at the beginning of the experiments.

Experiment	Body weight			Plasma	
	Initial	At 19th day	Gain	Nitrogen	Alkaline phosphatase
	gm.	gm.	gm.	mg. per cc.	units per 100 mg. N
Control	176.6 \pm 3.3*	193.5 \pm 4.4	16.9	9.37 \pm 0.23	1.19 \pm 0.12
Injected†	185.3 \pm 4.4	268.8 \pm 6.6	83.5	10.30 \pm 0.68	1.99 \pm 0.36

* Mean \pm standard deviation.

† The animals were receiving intraperitoneally 0.5 mg. of growth hormone daily for 20 days and were fasted 24 hours preceding autopsy.

It is well known that pituitary growth extracts exert specific effects on the epiphyseal cartilages of hypophysectomized rats (4, 5). Recent experiments of Becks *et al.* (6) have shown that the growth hormone was able to reawaken chondrogenic and osteogenic processes in the epiphyseal cartilage of the tibia in hypophysectomized rats, even after postoperative intervals of a year or longer, to an extent comparable to that seen in normal young, growing rats. Since alkaline phosphatase is indispensable in bone formation (7), the increase in the plasma alkaline phosphatase level following growth hormone injections might be assumed to be specifically related

to the changes provoked by the hormone in the bony structures. However, alteration in the enzyme level in the blood serum may also be due to processes other than those involved in bone formation. There are indications (7) that phosphatases are concerned with protein synthesis; since growth hormone reduces the urinary nitrogen (8) and causes an increase in body protein,³ the elevation of plasma alkaline phosphatase following the administration of growth hormone may be related to this characteristic of the hormone.

Previous studies have shown that, when adrenocorticotrophic hormone is injected simultaneously with the growth hormone in hypophysectomized

TABLE IV

Effect of Growth and Adrenocorticotrophic Hormones and Their Combination on Plasma Alkaline Phosphatase of Hypophysectomized Male Rats

Hormone	Daily dose*	No. of rats	Body weight			Plasma	
			Initial	Autopsy	Gain	Nitrogen	Alkaline phosphatase
	mg.		gm.	gm.	gm	mg. per cc	units per 100 mg. N
Growth	0.10	6	144.8 ± 5.2†	159.5 ± 0.47	14.7	8.55 ± 0.22	4.66 ± 0.47
Adrenocorticotrophic	0.50	4	145.7 ± 5.5	116.7 ± 6.4	-29.0	10.50 ± 0.35	2.87 ± 0.44
Growth	0.1	5	148.5 ± 5.8	134.3 ± 8.0	-14.2	11.46 ± 0.36	2.52 ± 0.30
Adrenocorticotrophic	0.5						

* Rats operated on at 40 days of age; intraperitoneal injections begun on the day of hypophysectomy once daily for 15 days except Sunday (thirteen injections).

† Mean ± standard deviation.

rats, a counteraction exists between these two hormones (9, 10). It is of interest to investigate whether this antagonism might also be found in the concentration of plasma alkaline phosphatase. It has been shown previously that adrenocorticotrophic hormone reduces the content of this enzyme in rat plasma (1). Table IV presents a summary of the data, indicating that there is indeed an antagonism occurring between growth and adrenocorticotrophic hormones. It may be seen in Table IV that when 0.10 mg. of growth hormone is injected daily in hypophysectomized male rats simultaneously with 0.50 mg. of adrenocorticotrophic hormone for 15 days, the alkaline phosphatase content is reduced from 4.66 units, the value obtained with growth hormone alone, to 2.52 units per 100 mg. of plasma N. Thus, the influence of growth hormone on alkaline phosphatase is completely masked by the action of adrenocorticotrophic hormone. If

³ Li, C. H., unpublished data.

the influence of growth hormone on the concentration of plasma phosphatase is regarded as indicative of the hormonal action on either bone formation or general body growth, or both, the observed counteraction of the growth and adrenocorticotrophic hormones in the phosphatase activity is not surprising. In fact, it may be anticipated that other unknown enzymic processes concerned with these physiological changes will turn out to be affected in an opposite manner by these two pituitary factors.

SUMMARY

Administration of growth hormone has been shown to produce a marked increase in the alkaline phosphatase content of the plasma in both hypophysectomized and normal rats. It has also been shown that adrenocorticotrophic hormone counteracts the effect of growth hormone on the plasma alkaline phosphatase in hypophysectomized animals. The significance of the results is discussed with reference to the characteristic action of the hormone on bone formation and protein catabolism.

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THE VITAMIN B₆ GROUP

XI. AN IMPROVED METHOD FOR ASSAY OF VITAMIN B₆ WITH *STREPTOCOCCUS FAECALIS**

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The initial demonstration of the multiple nature of vitamin B₆ (2) and much of the work leading to the identification of pyridoxal and pyridoxamine as members of this group of vitamins (3-5) were carried out with *Streptococcus faecalis* R as the test organism. This organism requires vitamin B₆ for growth under defined conditions, but in contrast to pyridoxal and pyridoxamine, which are highly active in promoting growth, pyridoxine is essentially inactive (4). This organism was therefore used, in conjunction with *Lactobacillus casei* and appropriate chemical procedures, to demonstrate the occurrence of pyridoxal and pyridoxamine in natural materials (5). It was soon observed that in its quantitative applications the assay led, in certain cases, to inconsistencies. Thus, application of the assay to a liver preparation gave a result higher than that obtained with *Saccharomyces carlsbergensis*, which responds almost equally to pyridoxal, pyridoxamine, and pyridoxine (5). Similar results were obtained with yeast samples by Melnick and coworkers (6), and this fact, coupled with an apparent lability of the growth-promoting substance to acid, led the latter workers to postulate the existence of a fourth member of the vitamin B₆ group. Others (7, 8) also have expressed dissatisfaction with the use of this organism in the assay of vitamin B₆.

The present paper describes the results of a detailed investigation of the use of *Streptococcus faecalis* for the determination of pyridoxamine and pyridoxal. The medium and procedure developed permit obtention of duplicable and accurate results. It can also be applied, when desired, to the determination of other vitamins required by this organism.

EXPERIMENTAL

Test Organism and Culture Medium—*Streptococcus faecalis* R (American Type Culture Collection No. 8043) was carried by monthly transfer in yeast-dextrose agar, with incubation at 30°. As a point of departure, the medium and technique described by Snell and Rannefeld (4) were used.

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Synthetic folic acid, at a level of 0.1 γ per tube, was used in place of the folic acid concentrate, and the other vitamins were added to twice the levels originally recommended. As a result of preliminary experiments, adenine sulfate, guanine hydrochloride, and uracil were omitted from the medium, since growth of the organism in the presence of these substances was sometimes slightly inhibited but never stimulated.

Because of the lability of pyridoxal when the medium is autoclaved, all B₆-containing supplements were sterilized separately and added aseptically to tubes previously autoclaved with enough water to bring the total volume to 5 cc. The double strength basal medium was autoclaved separately, and, when cool, 5 cc. aliquots were added aseptically to each tube.

Inoculum—The inoculum was prepared from a 20 to 24 hour culture of *Streptococcus faecalis* grown in 10 cc. of the sodium citrate medium described below, which was supplemented with 100 millimicrograms of pyridoxal hydrochloride. The cells were centrifuged, washed in 10 cc. of sterile saline, recentrifuged, and resuspended in 10 cc. of sterile saline. Each tube was inoculated with 1 drop of this heavy suspension. Satisfactory growth was obtained with much lighter inocula, but, when these were used, a sigmoidal curve was obtained with pyridoxal. The heavy inoculum is therefore recommended.

Effect of Citrate—Growth on the unmodified medium was poor and the response to added pyridoxamine dihydrochloride or pyridoxal hydrochloride was usually erratic. The replacement of the sodium acetate of this medium by 2 per cent of sodium citrate (9) resulted in greatly increased growth, although growth still tended to be erratic. Since citrate was superior to acetate as a buffer in this medium, it was used in all further experiments.

Effect of Autoclaving—In occasional experiments, the addition of a mixture of synthetic amino acids to the basal medium markedly stimulated growth. It was felt that the erratic behavior of the organism from one assay to another might result from variable destruction in the autoclave of an essential amino acid. An experiment was therefore designed to determine the effects of different autoclaving times.

Aliquots of the double strength basal medium were autoclaved for 3, 6, 9, 12, and 15 minutes at 15 pounds pressure. An unautoclaved aliquot was also sterilized by filtration. 5 cc. of medium treated in each way were added to one series of tubes containing 10 millimicrograms of pyridoxal hydrochloride in 5 cc. of autoclaved water and to another series, of which each tube contained in addition 1 mg. of each of twenty-one crystalline amino acids. All tubes were then inoculated and incubated as usual. The results are given in Table I.

The data show that to support growth the medium must be autoclaved; however, prolonged autoclaving renders the medium incapable of supporting maximum growth. In the presence of extra amino acids, no deleteri-

TABLE I
Growth of Streptococcus faecalis in Media Autoclaved for Various Lengths of Time

	Time autoclaved, 15 lbs. pressure					
	min. 0	min. 3	min. 6	min. 9	min. 12	min. 15
	Galvanometer reading*					
No additions.....	86	37	40	49	51	61
Amino acids added...	60	38	37	37	37	39

* Per cent transmission; uninoculated basal medium = 100.

TABLE II
Effect of Individual Amino Acids on Growth of Streptococcus faecalis in Unautoclaved and Autoclaved Media

Amino acid*	Galvanometer reading		Amino acid*	Galvanometer reading	
	Auto-claved†	Unauto-claved‡		Auto-claved†	Unauto-claved‡
No additions	76	80	DL-Isoleucine	79	85
DL-Alanine	75	82	L-Leucine	80	81
L-Alanine	76	81	L-Lysine	79	89
L-Arginine	75	73	DL-Methionine	79	84
Asparagine	77	77	DL-Phenylalanine	80	85
DL-Aspartic acid	78	80	L-Proline	78	76
L-Cystine	53	79	DL-Serine	79	80
L-Cysteine	43	43	DL-Threonine	79	96
Glycine	78	97	DL-Tryptophan	78	87
L-Glutamic acid	78	92	L-Tyrosine	77	84
L-Histidine	77	85	DL-Valine	78	77

* 2 mg. of DL-amino acid or 1 mg. of L-amino acid per 10 cc. of medium which contained 10 millimicrograms of pyridoxal hydrochloride.

† The medium was autoclaved 20 minutes at 15 pounds pressure.

‡ The medium was filtered through a frittered glass filter.

ous effects of prolonged autoclaving are observed, and considerable growth occurred in the unautoclaved medium.

To determine the amino acids effective in permitting growth under these conditions, each of twenty-one amino acids was tested individually in an unautoclaved medium and in a medium autoclaved for 20 minutes at 15 pounds pressure. The results are shown in Table II. Only cysteine

and cystine stimulated growth when added to the autoclaved medium' and only cysteine permitted maximum growth in the unautoclaved medium.

The effectiveness of these compounds and other reducing agents in permitting growth in unautoclaved and overautoclaved media is shown

TABLE III

Effect of Various Supplements on Growth of Streptococcus faecalis in Unautoclaved and Autoclaved Media

Additions	Amount	Galvanometer reading			
		Filtered*		Autoclaved*	
		Pyridoxamine dihydrochloride, millimicrograms			
		0	10	0	10
	mg				
None	0	100	91	96	62
	0	99	92	93	64
Autoclaved glucose†	5	97	75	93	61
	15	89	34	92	62
Ascorbic acid	1	90	40	92	64
	3	90	36	94	67
	5	90	32	91	65
Sodium thioglycolate	0.5	97	89	91	53
	1	97	74	90	50
	2	92	76	95	44
Cysteine hydrochloride	0.5	95	89	94	31
	1	97	92	94	31
	2	93	49	92	33
	4	93	47	94	34
Cystine “	0.5	100	92	92	32
	1	99	92	94	30
	2	100	92	93	31
	4	98	93	91	31

* Filtered = medium passed through frittered glass filter. Autoclaved = medium autoclaved 15 minutes at 15 pounds pressure.

† 0.1 gm. of dextrose and 2 cc. of 1 N NaOH in 10 cc. of water were autoclaved 15 minutes at 15 pounds pressure.

in Table III. Again very poor growth was obtained on the filtered medium. The addition of alkali-treated glucose or ascorbic acid to this medium permitted maximum growth; sodium thioglycolate and cysteine were effective to a lesser degree; cystine was ineffective. On the autoclaved medium, maximum growth was obtained only when cysteine or cystine was added. Alkali-treated glucose and ascorbic acid had no growth-promoting effect; sodium thioglycolate permitted increased, although not maximum, growth.

As a result of these findings, extra cystine has been added to the medium as follows: The double strength basal medium containing 1 mg. of cystine per 5 cc. is autoclaved for 10 minutes at 15 pounds pressure. When it has cooled, 0.33 cc. of a sterile solution of cystine hydrochloride (3 mg. per cc.) is added aseptically per 5 cc. of double strength basal medium. It is advisable to include some cystine in the basal medium during the autoclaving process, as well as to supply the additional quantity. If desired, a considerably larger quantity (3 to 4 mg.) of cystine may be added initially; sufficient escapes destruction to permit growth after any reasonable period of autoclaving.

TABLE IV
Composition of Basal Medium

Substance	Amount per 100 cc. double strength medium (20 assay tubes)	Substance	Amount per 100 cc. double strength medium (20 assay tubes)
	mg.		mg.
Acid-hydrolyzed casein*	1000	NaCl	2
Glycine	40	FeSO ₄ ·7H ₂ O	2
Asparagine	20	MnSO ₄ ·H ₂ O	2
DL-Tryptophan	20		γ
L-Cystine	20	Riboflavin	80
Dextrose	2000	Thiamine hydrochloride	40
Sodium citrate	4000	Calcium pantothenate	80
KH ₂ PO ₄	600	Niacin	80
K ₂ HPO ₄	600	p-Aminobenzoic acid	40
MgSO ₄ ·7H ₂ O	40	Folic acid	2
NaHCO ₃	20	Biotin	0.4

* Prepared from Labco "vitamin-free" casein, as described elsewhere (4).

With these modifications and additions, the poor and erratic results obtained with the original technique appeared to be largely overcome. The basal medium, as finally adopted, is given in Table IV. For convenience, the components of the medium were stored in three separate solutions: Solution A, the casein hydrolysate with added amino acids; Solution B, the sugar and inorganic salts; and Solution C, the vitamins. Solutions A and C were kept in a refrigerator under toluene, while Solution B was kept frozen in a refrigerator at -4° . Such solutions are stable for at least 1 to 2 months. The basal medium was prepared by mixing the calculated amounts of each of these solutions and adjusting the pH to 6.8 with NaOH.

Length of Incubation—In applying the modified medium to the assay of vitamin B₆ in natural materials, the length of incubation was found to

have a marked effect on the results obtained. A sample of a dried yeast was hydrolyzed for 5 hours with 0.055 N HCl (1) and assayed with *Streptococcus faecalis* with the modified medium described. The turbidities of the same tubes were determined after various time intervals. Results are shown in Fig. 1. It is seen that if the vitamin B₆ content were calculated before 20 hours incubation, the results would be a measure not only of the vitamin B₆ present, but also of other substances stimulatory to the growth of *S. faecalis*. The existence of such substances has been reported previously (10). Since they are stimulatory only, and not essential for growth, their effect disappears after longer incubation periods. In the

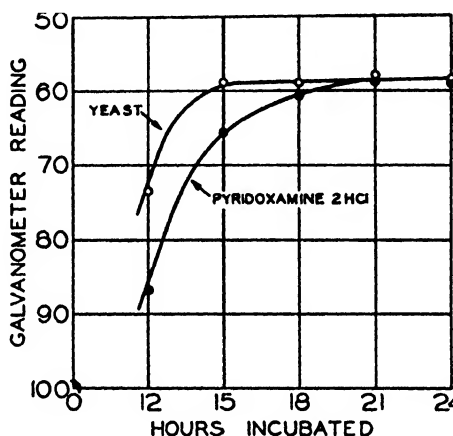


FIG. 1. The effect of time on the growth response of *Streptococcus faecalis* to equivalent amounts of a dried yeast (0.09 mg.) and to pyridoxamine dihydrochloride (4 millimicrograms) in the vitamin B₆-free medium.

assay of natural materials for vitamin B₆ with *S. faecalis*, therefore, turbidity should be determined only after 20 to 24 hours incubation.

The detailed procedure for determination of vitamin B₆, as modified in the present study, is therefore as follows: Enough water to give a volume of 5 cc., when the supplements have been added, is autoclaved 10 minutes at 15 pounds pressure in 18 × 150 mm. Pyrex test-tubes which are covered with aluminum caps. After the tubes have cooled, supplements, which have been diluted with sterile water are added to the tubes aseptically. Sufficient double strength basal medium for the assay is autoclaved in one flask for 10 minutes at 15 pounds pressure. When it has cooled, a previously autoclaved solution of L-cystine (3 mg. per cc.)¹ is added to

¹ To prepare this solution, suspend 0.5 gm. of L-cystine in a small amount of water, add concentrated HCl dropwise, with heating, until the solution is just effected; then dilute to 3 mg. of cystine per cc.

the basal medium in sufficient quantity to supply 1 mg. per 5 cc., as described earlier. A 5 cc. portion of the basal medium is then added aseptically to each tube. The tubes are inoculated with 1 drop of a heavy inoculum and placed in an incubator at 30° for 22 to 24 hours. At the end of this period they are heated in flowing steam at 100° for 10 minutes to stop growth. After cooling, the turbidities are determined with the Evelyn colorimeter with the 660 m μ filter.

Application of Assay to Natural Materials—The vitamin B₆ content of a number of natural materials was determined with *Streptococcus faecalis*,

TABLE V
Vitamin B₆ Content of Some Natural Materials Determined with *Saccharomyces carlsbergensis* and *Streptococcus faecalis*

Substance	Sample treatment*	Vitamin B ₆ content†	
		Yeast	<i>S. faecalis</i>
		γ per gm.	γ per gm.
Dried brewers' yeast‡	W.	57.4	46.7§
Difco yeast extract	"	25.8	17.5
Wilson's 1:20 liver powder	"	59.4	47.6
Cerophyl	S.	15.0	7.8
Vitab	"	153	14.0
Whole wheat flour	"	6.0	0.9

* W. = autoclaved 5 hours at 20 pounds pressure with 180 cc. of 0.055 N HCl; S. = autoclaved 5 hours at 20 pounds pressure with 10 cc. of 2 N HCl.

† The activities of pyridoxamine dihydrochloride, pyridoxal hydrochloride, and pyridoxine hydrochloride for *S. carlsbergensis* were 1.0, 1.3, and 1.2 respectively. The vitamin B₆ content of vitab and whole wheat flour, as determined with *S. carlsbergensis*, has also been expressed as pyridoxamine dihydrochloride, although it is realized that the major portion of the activity is due to pyridoxine. The activities of pyridoxamine dihydrochloride and pyridoxal hydrochloride for *S. faecalis* were 1.0 and 1.1 respectively.

‡ A sample of dried yeast, R. C. No. 6, kindly supplied by Dr. L. Atkin.

§ The average of sixteen separate determinations on different hydrolysates of the yeast sample. The average deviation was 4 γ per gm.

with this improved method, and compared with the values obtained on the same samples with *Saccharomyces carlsbergensis* as the test organism (1, 11). The results are shown in Table V.

All samples were hydrolyzed under conditions previously shown to liberate maximum amounts of vitamin B₆, as determined by the yeast assay (1). With the exception of dried yeast samples, which showed maximum vitamin B₆ activity for *Streptococcus faecalis* before acid hydrolysis (cf. (12)), the vitamin B₆ content of the natural materials tested was also unavailable to *Streptococcus faecalis*, unless the samples were acid-hydrolyzed. Prolonged acid hydrolysis did not destroy any of the vitamin.

The vitamin B₆ content of yeast, liver, and Cerophyl samples, as determined with *Saccharomyces carlsbergensis*, has been expressed as pyridoxamine dihydrochloride, instead of as pyridoxine hydrochloride, as has been done in previous investigations (5-7, 1), since the greater proportion of the vitamin B₆ of these substances is present as pyridoxamine.

It is evident from Table V that the vitamin B₆ content of all materials tested is smaller when determined with *Streptococcus faecalis* than when determined with *Saccharomyces carlsbergensis*. The activity of these samples, then, can be explained in terms of their pyridoxine, pyridoxamine, and pyridoxal content alone.

TABLE VI

Response of Various Strains of Streptococcus faecalis to Pyridoxamine, Pyridoxal, and Yeast Extract

Strain of <i>S. faecalis</i>	Galvanometer reading		Relative activity, pyridoxal to pyridoxamine*	Vitamin B ₆ in yeast (as pyridoxamine dihydrochloride)
	Pyridoxamine dihydrochloride, millimicrograms			
	0	10		
R	98	43	1.1 (0.6-1.3)	44
734	88	36	1.3 (1.2-1.4)	42
742	97	39	1.7 (1.5-1.8)	44
718	98	64	1.1 (0.4-1.7)	42

* In some cases, the ratio of activity of pyridoxal to pyridoxamine varies with the amount added; *i.e.*, the dose-response curves to the two substances are dissimilar. The limits of such variations are indicated by the figures in parentheses. The first such figure gives the activity at the lowest level, and the latter figure gives the activity at the highest level.

Other Strains of Streptococcus faecalis—A number of other strains of *Streptococcus faecalis*, obtained from Dr. J. M. Sherman, were used for the assay of vitamin B₆ in a dried brewers' yeast. The sample was autoclaved in 180 cc. of 0.055 N HCl for 5 hours at 20 pounds pressure, a treatment which has been shown to liberate maximum amounts of the vitamin from such a yeast sample when determined with *Saccharomyces carlsbergensis* (1). In each case the vitamin B₆ content of the yeast is the average of ten levels tested, containing from 0.5 to 5.0 millimicrograms of the vitamin. The results are given in Table VI.

Although the ratio of the activity of pyridoxamine dihydrochloride to pyridoxal hydrochloride varied greatly among the strains tested, the vitamin B₆ content of the sample, calculated as pyridoxamine dihydrochloride, was found to be the same, regardless of the culture used. This

indicates that pyridoxal contributes only a small portion of the vitamin B₆ activity of a yeast sample when prepared as described. Similar conclusions have been reached with *Lactobacillus casei*, which responds only to pyridoxal, as the test organism (5-7).

The extent of growth obtained on this medium also varied with each culture; yet the vitamin B₆ content of the natural sample was the same in all cases. This is added evidence for the reliability of the method finally adopted, and indicates that growth-stimulatory substances present in the sample do not interfere with the determination of vitamin B₆.

Use of Medium with Streptococcus faecalis for Other Vitamins—The improved medium may be used without modification in the assay of

TABLE VII

Response of Streptococcus faecalis R to Calcium Pantothenate, Niacin, Folic Acid, and Biotin in Modified Medium

Calcium pantothenate		Niacin		Folic acid		Biotin	
Milli-micrograms per tube	Galva-nometer reading	Milli-micrograms per tube	Galva-nometer reading	Milli-micrograms per tube	Galva-nometer reading	Milli-micrograms per tube	Galva-nometer reading
0	97	0	97	0	95	0.0	93
100	82	50	84	1	89	0.25	68
200	57	100	75	2	82	0.50	58
300	44	150	68	3	78	0.75	44
400	38	200	64	4	72	1.0	
600	33	350	50	5	69	2.0	41
800	30	400	51	8	59	3.0	36
1000	28	500	47	10	55	4.0	33

niacin, folic acid, and pantothenic acid. The charcoal treatment used in preparing the casein hydrolysate does not completely remove the biotin present. For this purpose, it is necessary to treat the casein with charcoal at pH 2.0 to 3.5 before it can be used for the assay of biotin.

The response of *Streptococcus faecalis* to added amounts of each of these vitamins on the modified medium from which each vitamin under test has been omitted individually is shown in Table VII. When used in this manner, 1 γ of pyridoxamine dihydrochloride or pyridoxal hydrochloride per 10 cc. of medium was used as a source of vitamin B₆.

DISCUSSION

The most obvious difficulties encountered in using the original medium described with *Streptococcus faecalis* were the comparatively poor growth and the erratic nature of the growth, both in a single assay and among

different assays. One factor responsible for the superior growth obtained in the present studies was the substitution of sodium citrate for sodium acetate. A comparison of the growth of *Streptococcus faecalis* on the modified medium and on this same medium in which the sodium citrate is replaced by 60 mg. of sodium acetate per tube is given in Fig. 2. With the modified medium and procedure, the curve obtained with sodium acetate is quite regular, although the growth is less extensive than when sodium citrate is used as a buffer.

The primary factor responsible for the erratic nature of the results, however, was the variable effect of autoclaving, which, although necessary

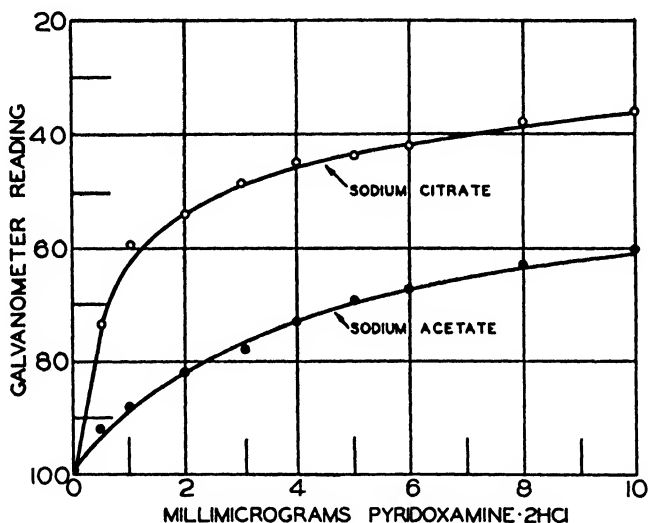


FIG. 2. A comparison of citrate and acetate as buffers for the growth of *Streptococcus faecalis*.

to permit growth, rendered the medium unsuitable for growth if continued for as long as 15 minutes. It is interesting to note that the fortuitous adoption of a favorable autoclaving time would permit regular results to be obtained with the earlier, unmodified medium. Such a circumstance, in fact, permitted extensive use of the earlier assay by one of us (2-5) without serious irregularities; these came to light when, on removal to another laboratory with a different type of autoclave, the original assay procedure proved almost useless.

Orla-Jensen (13), using a variety of lactic acid bacteria, demonstrated that most of these organisms would not grow on a yeast autolysate-glucose medium unless the glucose were autoclaved with the nitrogen source or in

basic solution. Smiley and coworkers (14), using a chemically defined medium, found that *Streptococcus salivarius* behaved in a similar manner. However, Niven and Sherman (15) found that *Streptococcus faecalis* would grow on an unautoclaved, synthetic medium which contained sodium thioglycolate.

The results obtained in this study indicate that failure of *Streptococcus faecalis* to grow in the unautoclaved medium is due to the lack of a suitable reducing agent, the requirement for which is comparatively non-specific, since it is satisfied by ascorbic acid, thioglycolic acid, cysteine, or glucose autoclaved under basic conditions. Failure to grow in the overautoclaved medium results from destruction of cystine or cysteine during autoclaving; after such treatment, the medium is rendered suitable for growth by the addition of extra cystine or cysteine.

SUMMARY

The assay method for vitamin B₆ (pyridoxal and pyridoxamine) which uses *Streptococcus faecalis* as the test organism (4) has been modified. The substitution of sodium citrate for sodium acetate permits much more extensive growth. If unautoclaved, the medium does not support the growth of *Streptococcus faecalis*; under these conditions, the addition of reducing agents such as ascorbic acid, sodium thioglycolate, cysteine, or alkali-treated glucose permits extensive growth. A medium autoclaved more than 6 minutes at 15 pounds pressure also does not support maximum growth of the organism, due to the destruction of cystine and cysteine. This effect is readily overcome by supplementing the autoclaved medium with cystine which had been sterilized separately, or by adding excess cystine before autoclaving. These modifications have largely eliminated the erratic and variable results obtained with the earlier method.

In applying the method to the determination of vitamin B₆, it is necessary to incubate for longer periods (20 hours) than previously thought necessary to eliminate the effect of non-essential growth stimulants present in natural materials.

With the modified procedure and with properly hydrolyzed extracts, the vitamin B₆ (pyridoxal plus pyridoxamine) content of natural materials obtained with *Streptococcus faecalis* were uniformly less than the values obtained with *Saccharomyces carlsbergensis* (pyridoxal, pyridoxamine, and pyridoxine). These results do not support, therefore, the hypothesis (6) that a fourth unidentified member of the vitamin B₆ group exists.

Four different strains of *Streptococcus faecalis*, which differed in their growth characteristics on the assay medium, gave identical values for the vitamin B₆ content of yeast extract.

Satisfactory standard curves for other vitamins required by *Streptococcus faecalis* are readily obtained with the medium; its application to their assay has not been investigated further.

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THE VITAMIN B₆ GROUP

XII. MICROBIOLOGICAL ACTIVITY AND NATURAL OCCURRENCE OF PYRIDOXAMINE PHOSPHATE*

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In most natural materials, vitamin B₆ occurs in a bound form, unavailable to organisms such as *Saccharomyces carlsbergensis* unless liberated by acid hydrolysis (2). For the latter purpose, hydrolysis with 0.055 N hydrochloric acid is considerably more effective than higher concentrations of acid (2, 3). This unusual behavior is shared by pyridoxal phosphate, which occurs naturally as a prosthetic group of transaminase (4) and amino acid decarboxylases (5), and which also shows only weak growth-promoting properties before hydrolysis. From these results, it might be concluded that the bound form of vitamin B₆ present in natural materials was pyridoxal phosphate. However, preliminary microbiological assays of natural materials indicated that most of the vitamin B₆ of yeast, liver powder, and similar materials was present as pyridoxamine rather than as pyridoxal or pyridoxine (6). These results were later verified with more adequate extraction procedures and a more satisfactory assay procedure (1). Similarly, direct assay showed the pyridoxal phosphate content of dried yeast to account for only 3 to 10 per cent of the total vitamin B₆ in the sample (3). These results show that the pyridoxal phosphate content of natural materials cannot fully explain their behavior toward acid hydrolysis.

This paper presents evidence to show that the principal bound form of vitamin B₆ in natural materials is *pyridoxamine phosphate*. The ability of synthetic pyridoxamine phosphate to function as a cotransaminase (but not as a codecarboxylase) has been reported (4). Colorimetric analyses have also indicated that this substance may occur naturally in some preparations of transaminase from pig heart (7). However, the specificity of the methods used to detect pyridoxamine in the latter investigation has been seriously questioned (8).

EXPERIMENTAL

Assay Procedures—Assays with *Streptococcus faecalis* were performed by the modified procedure described in the preceding paper (1). Assays

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with *Saccharomyces carlsbergensis* 4228 and *Lactobacillus casei* were performed by published methods (2, 9, 10).

Preparation and Microbiological Activity of Pyridoxamine Phosphate—Purified preparations of pyridoxamine phosphate are not available. The structure of this ester, like that of pyridoxal phosphate itself, is unknown. For use in the experiments described below, a solution of pyridoxamine phosphate was prepared by mixing 300 γ of a preparation of pyridoxal phosphate (supplied by Dr. Karl Folkers of Merck and Company, Inc.) with 30 mg. of L-glutamic acid adjusted to pH 6.9 with sodium hydroxide

TABLE I

Microbiological Activity of Pyridoxal Phosphate and Pyridoxamine Phosphate

	Activity					
	Pyridoxal phosphate* (pyridoxal hydrochloride = 1.0)			Pyridoxamine phosphate (pyridoxamine dihydrochloride = 1.0)		
	None	HCl†	NaOH‡	None	HCl†	NaOH‡
<i>Saccharomyces carlsbergensis</i> .	0.03	0.16	0.02	0.02	0.17	0.01
<i>Streptococcus faecalis</i> § ..	0.03	0.17	0.04	0.12-0.36	0.18	0.1-0.3
<i>Lactobacillus casei</i>		0.17		0.01	0.01	

* The pyridoxal hydrochloride content of this sample, when determined by the modified Scudi phenol method (12), was 0.19 after acid hydrolysis.

† 30 γ of vitamin B₆-phosphate + 10 cc. of 0.055 N HCl autoclaved 5 hours at 15 pounds pressure.

‡ 30 γ of vitamin B₆-phosphate + 10 cc. of 1 N NaOH autoclaved for 5 hours at 15 pounds pressure.

§ Since the dose-response curves for the unhydrolyzed pyridoxamine phosphate and pyridoxamine differ, the activity of the former cannot be expressed as a single value. These figures express the extremes of the observed activity, which increase as the amount of pyridoxamine phosphate is increased.

(total volume 2 cc.) and autoclaving at 15 pounds pressure for 1 hour. These conditions have previously been shown to effect quantitative conversion of pyridoxal to pyridoxamine (11).

That the postulated conversion of pyridoxal phosphate to pyridoxamine phosphate actually occurred as a result of the described treatment is shown by the microbiological activity of the product, as given in Table I.

It will be recalled that for *Lactobacillus casei* only pyridoxal shows high activity, whereas for *Streptococcus faecalis* and *Saccharomyces carlsbergensis* both pyridoxal and pyridoxamine are highly active (10). Pyridoxal phosphate has very low activity for all three organisms but shows the expected activity, due to pyridoxal, for all three organisms after hydrolysis with acid (2). These results are confirmed by the present data.

Like pyridoxal phosphate (and unlike pyridoxal), the product of the above reaction (pyridoxamine phosphate) is largely unavailable to *S. carlsbergensis* and *L. casei*. In contrast to pyridoxal phosphate, however, the product shows high activity for *S. faecalis*, which varies from somewhat less to somewhat greater than that of pyridoxamine as the testing level is increased. Hydrolysis of the product with acid yields pyridoxamine, highly active for *S. carlsbergensis* and *S. faecalis*, but essentially inactive for *L. casei*. It should be noted that (a) the conversion is essentially quantitative, the amount of pyridoxamine obtained by hydrolysis of the reaction product equaling the amount of pyridoxal obtained by hydrolysis

TABLE II
Vitamin B₆ Content of Dried Brewers' Yeast*

Hrs. hydrolysis	Vitamin B ₆ content† (γ per gm ± average deviation of points in single assay)		
	<i>Saccharomyces carlsbergensis</i>	<i>Streptococcus faecalis</i>	<i>Lactobacillus casei</i>
0	6.7 ± 0.2	41.7 ± 10.0	0.6
1	33.7 ± 1.3	44.5 ± 5.9	3.8
3	46.5 ± 1.7	43.8 ± 0.9	3.3
5	49.2 ± 1.4	40.6 ± 0.9	4.3
0-5†	43.4 ± 1.1	42.7 ± 0.8	

* A sample R. C. No. 6 kindly supplied by Dr. L. Atkin.

† Expressed as pyridoxamine dihydrochloride for the *S. carlsbergensis* and *S. faecalis* assay and as pyridoxal hydrochloride for the *L. casei* assay. The relative activities of pyridoxamine dihydrochloride, pyridoxal hydrochloride, and pyridoxine hydrochloride for *S. carlsbergensis* are 1.0, 1.3, and 1.2 respectively. The activities of pyridoxamine dihydrochloride and pyridoxal hydrochloride for *S. faecalis* were 1.0 and 1.1 respectively.

‡ A water extract, prepared by filtering a water suspension of the yeast, acidified with HCl to 0.055 N and autoclaved 5 hours at 20 pounds pressure.

of the starting material, and that (b) no dephosphorylation accompanies the reaction, as evidenced by the inactivity of the product for *S. carlsbergensis*. Like pyridoxal phosphate, the product is stable to alkaline hydrolysis, but is hydrolyzed by acid.

A similar procedure for preparation of pyridoxamine phosphate was employed by Umbreit *et al.* (4); the product was ineffective as a coenzyme for amino acid decarboxylation but retained activity as cotransaminase.

Natural Occurrence of Pyridoxamine Phosphate—Previous results showed that for maximal release of vitamin B₆ from a dried brewers' yeast, 5 hours hydrolysis at 20 pounds pressure with 0.055 N hydrochloric acid was necessary when *Saccharomyces carlsbergensis* was the test organism. The effect of varying the time of hydrolysis on the apparent vitamin B₆ content of this yeast, as determined with three organisms, is shown in Table II.

The values obtained with *Lactobacillus casei* as the test organism show clearly that the amount of pyridoxal phosphate present is insufficient to explain the large increase in activity observed with *Saccharomyces carlsbergensis* on prolonged acid hydrolysis.

The results obtained with *Saccharomyces carlsbergensis* are very similar to those already reported (6), and demonstrate the necessity for adequate hydrolytic treatment in the determination of the vitamin B₆ content of

TABLE III
Vitamin B₆ Content of Natural Materials after Various Hydrolytic Treatments

Test organism	Substance	Pyridoxamine dihydrochloride per gm			
		Hydrolytic treatment			
		None*	HCl†	NaOH‡	NaOH-HCl§
		γ	γ	γ	γ
<i>Saccharomyces carlsbergensis</i>	Dried brewers' yeast	6.7	57.4	5.4	36.2
	Wilson's 1:20 liver powder	20.8	59.4	17.0	46.0
	Cerophyl	4.5	15.0	2.6	11.1
<i>Streptococcus faecalis</i>	Dried brewers' yeast	41.7	46.7	41.3	39.4
	Wilson's 1:20 liver powder	3.3	47.6	35.5	38.7
	Cerophyl	4.5	8.4	5.1	8.0

* A water extract prepared by shaking the sample with water in a stoppered graduate, and filtering.

† Sample containing approximately 2 γ of vitamin B₆ autoclaved in 180 cc. of 0.055 N HCl for 5 hours at 20 pounds pressure, neutralized, diluted, and filtered.

‡ 50 mg. sample autoclaved in 10 cc. of 1 N NaOH for 30 minutes at 20 pounds pressure, neutralized, diluted, and filtered.

§ 50 mg. sample autoclaved in 10 cc. of 1 N NaOH for 30 minutes at 20 pounds pressure, neutralized, and diluted to 100 cc. 50 cc. of this plus 0.28 cc. of 10 N HCl (to make the final acidity 0.055 N) autoclaved for 12 hours at 20 pounds pressure, neutralized, diluted, and filtered.

natural materials. The fact that the vitamin B₆ content of an acid-hydrolyzed water extract of this sample was near the maximum obtained by acid hydrolysis of the whole yeast shows that the vitamin B₆ in this yeast occurs in a water-soluble combined form which is unavailable to *Saccharomyces carlsbergensis* unless hydrolyzed. This combined form is highly active for *Streptococcus faecalis*, as evidenced by the high values obtained for the vitamin B₆ content of the unhydrolyzed yeast sample. The large average deviation reflects the large "drift" which was always observed in assaying the unhydrolyzed sample. According to the amount

of sample assayed, the vitamin B₆ content, expressed as pyridoxamine dihydrochloride, varied from 20 γ per gm. at low levels (0.02 mg.) to 60 γ per gm. at higher levels (0.1 mg.). However, after acid hydrolysis the "drift" was no longer obtained.

The properties of this soluble substance thus far observed are identical with those of pyridoxamine phosphate, described above. Both substances

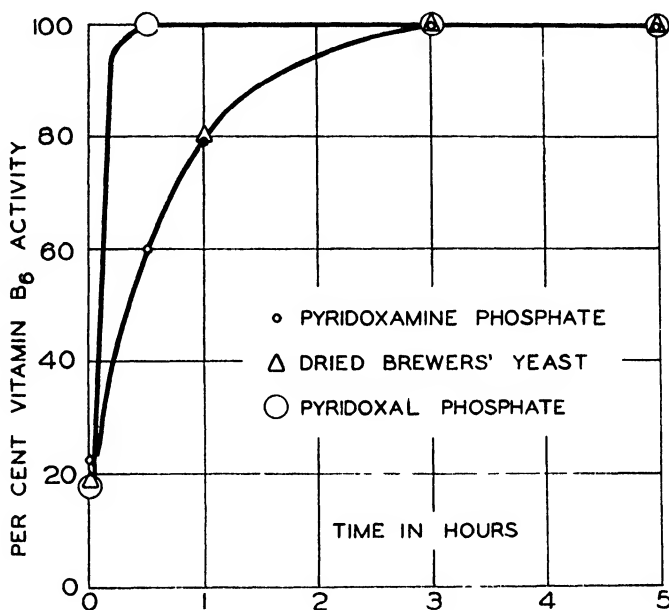


FIG. 1. The rate of liberation of vitamin B₆ by acid hydrolysis from pyridoxal phosphate, pyridoxamine phosphate, and a dried yeast, as determined with *Saccharomyces carlsbergensis*.

are comparatively inactive for *Saccharomyces carlsbergensis* and *Lactobacillus casei* but are highly active for *Streptococcus faecalis*; on acid hydrolysis, both substances are active for *Saccharomyces carlsbergensis* and *Streptococcus faecalis*, but are inactive for *Lactobacillus casei*. The response of *Streptococcus faecalis* to both substances shows the same characteristic upward "drift" when compared with the response to pyridoxamine.

In Wilson's 1:20 liver powder and in Cerophyl, a grass product, water treatment did not extract this substance. On alkali treatment, to which both pyridoxal phosphate and pyridoxamine phosphate are stable, the same substance, showing high activity for *Streptococcus faecalis* but little for *Saccharomyces carlsbergensis*, was extracted from these materials as well (Table III). On hydrolysis of the alkaline extract with acid, con-

siderable vitamin B₆ (pyridoxamine), active for both *Saccharomyces carlsbergensis* and *S. faecalis*, was liberated. The unhydrolyzed substance shows, therefore, the same stability to alkali as pyridoxamine phosphate.

It will be noted that the vitamin B₆ content of unhydrolyzed Wilson's liver powder obtained by assay with *S. carlsbergensis* is significantly higher than that obtained with *S. faecalis*. This is ascribed to the presence of some free pyridoxine, which is fully active for *S. carlsbergensis*, but is inactive for *S. faecalis*.

Further evidence for the identity of this naturally occurring vitamin B₆ conjugate with pyridoxamine phosphate is provided by a comparison of the rates of hydrolysis of pyridoxamine phosphate and the rate of liberation of vitamin B₆ from the yeast sample by acid hydrolysis, shown in Fig. 1. *Saccharomyces carlsbergensis* was the test organism. 30 γ samples of pyridoxal or pyridoxamine phosphate in 10 cc. of 0.055 N HCl and 50 mg. samples of yeast in 180 cc. of 0.055 N HCl were autoclaved at 20 pounds pressure for the length of time indicated.

Pyridoxal is liberated from pyridoxal phosphate much more rapidly than pyridoxamine is liberated from pyridoxamine phosphate. The rate of liberation of pyridoxamine from pyridoxamine phosphate, however, is identical with the rate of liberation of vitamin B₆ from dried yeast.

DISCUSSION

Melnick and coworkers (12) recently concluded that, in addition to pyridoxal, pyridoxamine, and pyridoxine, a fourth unidentified member of the vitamin B₆ group must exist. Their evidence consisted chiefly of the following observations: (a) The vitamin B₆ content of yeast following hydrolysis with 2 N sulfuric acid was less than the value obtained after hydrolysis with 0.055 N sulfuric acid. Since pyridoxal, pyridoxamine, and pyridoxine are stable to 2 N acid, existence of an acid-labile form of the vitamin appeared probable. It has since been shown that on *prolonged* hydrolysis with 2 N acid, maximal values for vitamin B₆ can be obtained; the higher values obtained in short hydrolysis periods with 0.055 N acid result from the unusual circumstance that the low concentration of acid is a more effective hydrolytic agent than more concentrated acid for the naturally occurring bound form of vitamin B₆ (2, 3). (b) The vitamin B₆ content of yeast, as determined with *Streptococcus faecalis*, which responds only to pyridoxal and pyridoxamine was higher than that obtained with *Saccharomyces carlsbergensis*, which responds to pyridoxal, pyridoxamine, and pyridoxine. The existence of a compound with higher activity for *S. faecalis* than for *S. carlsbergensis* therefore appeared probable. Several explanations for obtention of high results with *S. faecalis* by these workers can now be given. Perhaps of most importance is the fact that the ex-

tracts assayed were not completely hydrolyzed. They therefore contained pyridoxamine phosphate, inactive for *S. carlsbergensis*, but active for *S. faecalis*; consequently higher results were obtained with the latter organism (cf. the same type of result with unhydrolyzed samples in Tables II and III). A contributory factor to such high results was undoubtedly the assay method used with *S. faecalis*. The preceding paper (1) describes various deficiencies encountered with this early method (failure to grow if the medium is insufficiently autoclaved or overautoclaved; response to stimulatory materials during short incubation periods, etc.), all of which would tend to produce erroneously high assay results with natural materials. With suitable modification, and with completely hydrolyzed samples, values obtained with *S. faecalis* for the vitamin B₆ content of natural materials are uniformly lower than those obtained with *S. carlsbergensis* (1). At present, therefore, no observations are available which require postulation of an additional member of the vitamin B₆ group for their explanation.

SUMMARY

When heated with glutamic acid under appropriate conditions, pyridoxal phosphate is converted quantitatively to a substance which appears to be pyridoxamine phosphate. This conversion is completely analogous to the known reaction which occurs with pyridoxal under similar conditions (11).

Like pyridoxal phosphate, pyridoxamine phosphate is much less active before than after hydrolysis for *Saccharomyces carlsbergensis* and *Lactobacillus casei*. However, for *Streptococcus faecalis* pyridoxamine phosphate is from 0.7 to 2 times as active before hydrolysis as after acid hydrolysis. Pyridoxamine phosphate is stable in sodium hydroxide at 120° for 5 hours. Acid hydrolysis of pyridoxamine phosphate liberates pyridoxamine; under the conditions tested, this hydrolysis occurs more slowly than that of pyridoxal phosphate.

A substance was found in dried yeast, liver powder, and a dried grass with properties similar in all respects to those of pyridoxamine phosphate. It occurs free in the dried yeast, since its presence can be demonstrated in a cold water extract of the sample. It may be extracted from liver and grass powders by treatment with alkali, which releases the phosphorylated vitamin without hydrolyzing it. On hydrolysis with weak acid, vitamin B₆ is liberated from dried yeast at a rate identical with the release of pyridoxamine from pyridoxamine phosphate. This is considered further evidence for the identity of the major portion of the vitamin present with pyridoxamine phosphate.

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STUDIES ON PHENYLPYRUVIC OLIGOPHRENIA*

THE POSITION OF THE METABOLIC ERROR

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Phenylpyruvic oligophrenia is a disease characterized clinically by mental defect and biochemically by the presence in the urine of phenylpyruvic acid (1-3) and phenylalanine (4, 5). In addition, abnormally high amounts of phenylalanine are found in the blood (4, 6).

Although there is little doubt that the condition is an error of metabolism of phenylalanine, which is inborn and inherited as a recessive Mendelian character (7, 8), the exact nature of the metabolic abnormality is still imperfectly known. It was the purpose of the investigation here reported to determine at what stage in the metabolic pathway of phenylalanine the error occurs.

Evidence will be presented which indicates that in the fasting normal organism the ingestion of phenylalanine is followed by a significant increase of substances in the blood giving a Millon reaction, while in patients suffering from phenylpyruvic oligophrenia no increase takes place.

This finding seems to indicate that the metabolic error which is at the base of phenylpyruvic oligophrenia lies in the inability normally to bring about the hydroxylation of phenylalanine.

EXPERIMENTAL

Adult rabbits, weighing from 2 to 3 kilos, and adult cats, weighing from 3 to 4 kilos, were used as experimental animals. All animals were kept fasting for 36 hours before and during the experiment.

The following substances were given by stomach tube: DL-phenylalanine (Winthrop), L-tyrosine (Merck), and phenylpyruvic acid. The dose was 0.5 gm. per kilo of body weight. Tyrosine was neutralized with sodium hydroxide; phenylalanine and phenylpyruvic acid were dissolved in adequate amounts of hot water and fed as acids. Blood was obtained by cardiac puncture immediately before the ingestion of the compound and at hourly intervals thereafter.

The human subjects were adult normal individuals and patients suffering from phenylpyruvic oligophrenia. They were kept fasting 15 hours before and during the experiment. DL-Phenylalanine (Winthrop), L-

* Aided by a grant from Child Neurology Research (Friedsan Foundation).

tyrosine (Merck), and phenylpyruvic acid were given by mouth. The dose was 5 gm.; the subjects weighed from 60 to 80 kilos. In three patients who were children, weighing from 20 to 30 kilos, the dose was 4 gm. Finally as a further control, glycine was fed in a dose of 10 gm. to normal individuals and to patients in order to determine whether an increase in Millon-reacting substances could be obtained. Blood was obtained by venipuncture at the fasting level and at half hour intervals for the first 2 hours following the ingestion of the compound, then at hourly intervals.

In each sample of blood from both experimental animals and human subjects, the Millon reaction was performed according to the directions of Bernhart and Schneider (9) by means of a Klett-Summerson electrophotometer with Filter 54. All values are expressed in mg. of tyrosine per 100 cc. of blood.

TABLE I
Millon Reaction in Blood of Animals Following Ingestion of DL-Phenylalanine

	Fasting	1 hr	2 hrs.	3 hrs.	4 hrs	5 hrs.
	mg per cent	mg per cent	mg per cent	mg. per cent	mg per cent	mg per cent
Rabbit 1	1.16	3.75	3.93	3.57	3.39	1.33
" 2	1.34	4.15	5.71	3.54	3.57	
" 3	1.43	4.20	3.93	3.39	2.76	2.76
" 4	1.87	4.00	6.23	5.71	4.46	
Cat 1	4.82	10.17	9.37	7.85		4.55
" 2	3.13	5.70	5.70	6.53	5.18	
" 3	3.65	5.71	7.23	6.21	4.14	
" 4	3.02	3.92	7.78	5.00	4.46	3.50

Samples of the phenylalanine and phenylpyruvic acid used in the experiments were tested for Millon-reacting substances with negative results.

Results

Table I shows the effect of the ingestion of DL-phenylalanine upon the Millon-reacting substances of the blood in cats and rabbits. An increase of tyrosine-like substance is clearly seen in every instance after 30 minutes, which reaches a maximum in 1 to 2 hours and declines thereafter.

Table II shows increases of Millon-reacting substances in the blood of two rabbits after the feeding of phenylpyruvic acid. There is a close similarity between the curves obtained following ingestion of phenylalanine and phenylpyruvic acid.

Table II shows also the effect of the ingestion of tyrosine upon the Millon-reacting substances in the blood of the cat and rabbit. As was to be expected, marked increases were noted.

In Table III the results of feeding phenylalanine to normal human subjects are reported. It will be seen that these figures are in accord with those obtained in experimental animals. An increase of Millon-reacting substances, which reaches a maximum in about 2 hours, was observed in every instance.

Table IV shows the effect of feeding phenylalanine to ten patients afflicted with phenylpyruvic oligophrenia. The results are clear cut.

TABLE II
Millon Reaction in Blood of Animals Following Ingestion of Phenylpyruvic Acid and L-Tyrosine

		Fasting	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.
		mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Phenylpyruvic acid	Rabbit 1	1.78	5.02	7.48	5.35	4.46	3.02
	" 2	1.96	4.19	2.67	1.96	2.07	2.07
Tyrosine	"	1.87	11.60	15.17	13.39	10.35	4.14
	Cat	4.46	24.10	12.70	6.76	4.14	4.10

TABLE III
Millon Reaction in Blood of Normal Human Subjects Following Ingestion of DL-Phenylalanine

Subject	Fasting	½ hr	1 hr.	1½ hrs.	2 hrs.	3 hrs	4 hrs.	5 hrs.
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
G. C.	0.98	2.07	2.14	2.23	1.96	1.62	1.58	
W. W.	0.98	1.34	1.46	1.54	2.05	1.70	1.61	1.34
C. D.	1.78	2.41	2.77	2.77	2.50	1.96	1.87	1.69
A. S.	1.03	1.52	1.87	1.96	2.77	2.23	1.87	
F. P.	0.98	1.42	2.08	1.96	1.69	1.78	1.42	
G. W.	0.80	1.16	1.42	1.51	1.27	1.16	1.03	
Average..	1.09	1.65	1.96	1.99	2.04	1.74	1.56	

In no instance was there observed a significant increase of Millon-reacting substances.

As is seen in Table V, following ingestion of phenylpyruvic acid, in a normal control there was an increase of Millon-reacting substances, while in a patient no increase was noted.

In Table VI are reported the values of Millon-reacting substances in the blood of both normal and phenylpyruvic individuals following the ingestion of 5 gm. of tyrosine. The increases observed in normal individuals confirm the results published by Bernhart and Schneider (9). It

can be seen that there is no significant difference in this respect between normal subjects and patients.

TABLE IV
Millon Reaction in Blood of Patients Following Ingestion of DL-Phenylalanine

Patient	Fasting	$\frac{1}{2}$ hr.	1 hr.	1 $\frac{1}{2}$ hrs	2 hrs.	3 hrs.	4 hrs.	5 hrs.
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
J. S.	0.98	0.88	0.88	0.98	0.88	0.88	0.88	0.88
R. G.	0.80	0.88	0.80	0.88	0.80	0.88	0.88	0.80
W. K.	0.88	0.98	0.88	0.88	0.88	0.80	0.80	0.80
E. A.	0.98	0.98	1.03	0.98	1.03	1.16	1.03	
A. D.	1.27	1.16	1.16	1.16	1.03	1.03	1.04	
J. B.	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.98
L. F.	0.98	0.88	0.88	0.98	0.98	0.88	0.88	0.88
W. R.	0.80	0.88	0.80	0.88	0.88	0.88	0.80	
B. C.	0.71	0.71	0.71	0.80	0.80	0.80	0.80	
R. K.	0.80	0.80	0.80	0.80	0.88	0.88	0.80	
Average	0.91	0.90	0.88	0.92	0.90	0.91	0.88	

TABLE V
Millon Reaction in Blood of Normal Human Subject and Patient Following Ingestion of Phenylpyruvic acid

	Fasting	$\frac{1}{2}$ hr	1 hr.	1 $\frac{1}{2}$ hrs.	2 hrs	3 hrs.	4 hrs.
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Control, F. P.	0.88	1.04	1.04	1.27	1.96	1.34	1.16
Patient, B. C.	0.80	0.80	0.72	0.80	0.80	0.71	0.80

TABLE VI
Millon Reaction in Blood of Normal Human Subject and Patients Following Ingestion of L-Tyrosine

	Fasting	$\frac{1}{2}$ hr.	1 hr.	1 $\frac{1}{2}$ hrs.	2 hrs.	3 hrs	4 hrs.	5 hrs.
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Control, L. O.	1.50	4.19	6.42	5.35	4.82	4.64	4.10	
Patient, B. C.	1.16	7.20	6.78	5.30	3.75	3.31	2.50	1.69
" W. C.	1.33	3.92	5.24	5.53	5.80	5.26	4.14	3.38

The feeding of glycine resulted in no increase of Millon-reacting substance in the blood of either normal or phenylpyruvic individuals.

Comment

The demonstration that in the fasting normal animal and human subject the ingestion of phenylalanine is followed by an increase of Millon-reacting substances is in accord with and in confirmation of the current theory that phenylalanine is normally catabolized through tyrosine.

In experiments, which are not reported in detail here, it could be demonstrated that the intravenous administration of phenylalanine to rabbits is followed also by increases of the tyrosine-like substance in the blood. This would indicate that the increases are not due to changes occurring in the gastrointestinal canal.

That the feeding of phenylpyruvic acid results also in increased Millon values of the blood can be explained readily, on the basis of the finding previously reported (6), that the organism is able to aminate this keto acid to form phenylalanine.

In the present experiments, it would have been desirable to isolate from the blood the tyrosine or the substance giving the Millon reaction, but the isolation of such small quantities involves technical difficulties which could not be overcome. It could be proved, however, that the substance which was responsible for the increase of the Millon reaction was not extracted by ether and was, therefore, not *p*-hydroxyphenyllactic or *p*-hydroxyphenylpyruvic acid.

The demonstration that hydroxylation of phenylalanine in the para position can be measured in the normal subject offers a method by which this metabolic step can be investigated in phenylpyruvic oligophrenia. The position of the metabolic block of phenylalanine in this disease is not known. Fölling *et al.* (8) advanced the hypothesis that the error consists in an abnormal racemization of phenylalanine, the naturally occurring L form being converted by the diseased organism into the D form which is subsequently decaminated in the kidney and excreted as phenylpyruvic acid. They were unable, however, to substantiate this hypothesis by finding the D isomer in the blood of patients.

Penrose and Quastel (10) claimed that the error is to be found in a block at the stage of phenylpyruvic acid, the patient being unable to break down the benzene ring of this keto acid. However, the presence of phenylalanine and the absence of phenylpyruvic acid in the blood of patients, together with the ability of the patients to aminate phenylpyruvic acid (6), are findings which cannot be explained by this hypothesis.

The data here presented, showing that no increase of Millon-reacting substances occurs in the blood of patients on administration of phenylalanine, indicate that the organism of the patient is unable normally to bring about the hydroxylation of phenylalanine. It seems, therefore,

that the biochemical error which is at the basis of phenylpyruvic oligophrenia consists of a block in the conversion of phenylalanine into tyrosine.

The finding that there is no significant difference between normal subjects and patients in their ability to dispose of tyrosine appears to indicate that the error is limited to the first stage of the metabolism of phenylalanine.

SUMMARY

Evidence is presented which indicates that in the normal fasting organism the ingestion of phenylalanine or of phenylpyruvic acid is followed by a significant increase of Millon-reacting substance in the blood. In patients suffering from phenylpyruvic oligophrenia, no increase of Millon-reacting substance is observed. It is, therefore, concluded that the biochemical error which is at the base of phenylpyruvic oligophrenia consists of a block in the normally occurring process of hydroxylation of phenylalanine in the para position.

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AN ELECTROPHORETIC STUDY OF PLASMA AND PLASMA FRACTIONS OF NORMAL AND INJURED RATS*

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The development of the electrophoretic procedure has led a number of investigators to study the distribution of rat plasma proteins by this method. Rat serum or plasma does not yield the distinct patterns that are obtained with human plasma. Moore, Levin, and Leatham (1) could not observe the α -globulin fraction in normal rat serum dialyzed against phosphate buffer at pH 7.4. After hypophysectomy (1) and thyroidectomy or thiouracil feeding in rats (2), the α -globulin appeared in the serum (pH 7.4). When normal rat serum was examined at pH 8.5, the α -globulin component was seen. Deutsch and Goodloe (3) found no distinct component in the region of the α -globulin at pH 8.6. Although five components have been described (1, 4), the boundaries are too indistinct for accurate analysis.

Experiences with the electrophoretic analysis of normal rat plasma in this laboratory were unsatisfactory and were similar to those of other investigators. In order to study the protein components more thoroughly, fractionation was undertaken according to the procedures recommended by Cohn and his associates (5). The present investigation is concerned with the analyses of whole and fractionated plasma obtained from normal and injured rats.

Methods

Inbred male rats of Wistar stock, about 70 days old and weighing between 150 and 200 gm., were used as experimental animals. About one-third of the body surface was burned by dipping the clipped back of the anesthetized rat in hot water at 75° for 40 seconds. These animals had access to food and water and were sacrificed at varying periods after being scalded. The animals were anesthetized with sodium pentobarbital and were exsanguinated by drawing blood from the aorta into a tube containing heparin.

Plasma was diluted with 1.5 parts of veronal buffer ($\Gamma/2$, 0.1) at pH 8.6

* This work was done under contract with the Medical Division of the Chemical Corps.

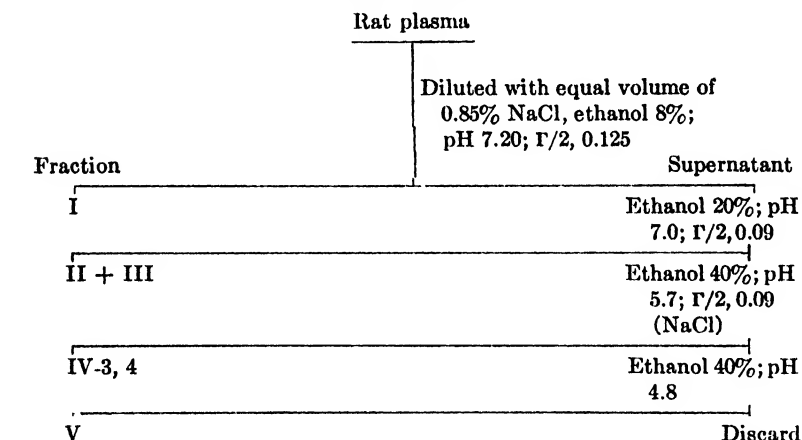
and dialyzed against the buffer for 3 or 4 days. The electrophoretic experiments were done in a Tiselius apparatus at 2.0° by means of the scanning method of Longworth (6). The concentrations of the various protein fractions were estimated from the patterns of the ascending side.

Results

Diagram 1 outlines the procedures used for fractionation of rat plasma. The extent of the fractionation was limited by the amount of plasma available (60 to 70 ml.). The four main fractions obtained were Fractions I, II + III, IV-4, and V.

DIAGRAM 1

Fractionation of Rat Plasma



All fractions were centrifuged at -5° for 40 minutes at 3000 R P M

Preliminary experiments showed that the dilution of plasma with 0.85 per cent NaCl is an important initial step. This dilution not only yields a larger volume for manipulation but the subsequent fractionation is more satisfactory. The ionic strength maintained with NaCl is more satisfactory than with sodium acetate. If the ionic strength of Supernatant II + III is increased above 0.09, the proteins of Fraction IV-4 become insoluble. Evidently an ionic strength greater than 0.09 at an alcohol concentration of 40 per cent causes some denaturation of one of the α -globulins which is present in Fraction IV-4.

Mobility Data—The mobilities of the various components are shown in Table I. The data are determined in whole plasma and its fractions of control and injured rats. The results indicate that the values for a given

TABLE I

Mean Values and Standard Errors for Mobilities of Proteins of Whole Plasma and Fractions

	Albumin	α_1	α_2	β	ϕ	γ
Ascending						
Plasma	6.6 ± 0.05	5.9 ± 0.05	5.0 ± 0.08	3.8	3.3 ± 0.04	2.2 ± 0.06
Fraction II + III	6.8 ± 0.07	6.0 ± 0.06	4.6 ± 0.06	3.8 ± 0.04		2.6 ± 0.06
Fraction IV-4	6.8 ± 0.05	6.0 ± 0.04	5.0 ± 0.07	3.8 ± 0.05		2.5 ± 0.10
Fraction V	6.7 ± 0.07	5.9 ± 0.05	4.5 ± 0.16	3.5 ± 0.05		
Descending						
Plasma	6.2 ± 0.07	5.4 ± 0.08	4.4 ± 0.07	3.0 ± 0.04	2.5 ± 0.07	1.9
Fraction II + III	6.7 ± 0.13	5.6 ± 0.11		3.4 ± 0.09		2.3 ± 0.03
Fraction IV-4	6.3 ± 0.04	5.4 ± 0.07	4.3 ± 0.07	3.2 ± 0.07		
Fraction V	6.3 ± 0.14	5.3 ± 0.06		3.1 ± 0.04		

TABLE II

Electrophoretic Analyses of Rat Plasma and Sera

Run No.	Treatment	Days after injury	Per cent distribution				
			Albumin	α_1	α_2	$\beta + \phi$	γ
102*	Normal		42	29	5	18	6
105	"		44	24	8	21	3
78	"		45	26		20	9
84	Burned	$\frac{1}{2}$	40	28	5	21	6
120	"	$\frac{1}{2}$	33	24	10		33
85	"	$\frac{3}{4}$	38†	25	12		25
75	"	1		52	16	29	3
103*	"	1	36	23	13	22	6
103	"	1	32	21	11	29	7
43	"	2	27	25	10		38
74	"	3		52	18		30
48	"	4	24	29	15		32
76	"	5		50	15		35

* Serum.

† Descending boundary.

component are the same regardless of methods of fractionation. The values for the descending boundaries are lower than those of the ascending boundaries, as is to be expected.

Electrophoretic Analysis—The electrophoretic analyses of plasma and serum and Fractions II + III, IV-4, and V are shown in Tables II to V for control and injured animals. Patterns for Fraction I were impossible to obtain.

The plasma of control rats is composed chiefly of albumin and α_1 - and β -globulins. After the animal is burned, the percentage distribution of albumin decreases and the α_2 -globulin increases. A study of whole rat plasma gives a rough approximation of the distribution of the protein components.

TABLE III
Electrophoretic Analyses of Fraction II + III

Run No.	Treatment	Days after injury	Albumin	α_1	α_2	β	γ	$\alpha_1:\gamma$	$\beta:\gamma$
			per cent	per cent	per cent	per cent	per cent		
44	Normal		21		14	11	54	0.26	0.20
46	"		9	17	11	20	43	0.25	0.46
49	"		7	14	14	12	53	0.26	0.22
135	"		5	15	8	17	55	0.15	0.31
120	Burned	$\frac{1}{2}$	8	24	18	28	22	0.82	1.27
75	"	1	7*	22	28	17	26	1.08	0.65
57	"	3	5	20	29	30	16	1.80	1.87
66	"	3	8	16	22	28	26	0.84	1.07
74	"	3	2	24	21	30	23	0.91	1.30
58	"	4	6	22	28	30	14	2.00	2.14
67	"	4	7	21	19	30	23	0.82	1.30
76	"	5	4	15	22	37	22	1.00	1.70
77	"	5	6	17	21	37	19	1.10	1.94

* Descending boundary.

Fraction I is composed chiefly of fibrinogen which on standing is converted to fibrin. Extracts of this fraction with barbiturate buffer were studied electrophoretically and it was found that about 60 per cent of the soluble material was α -globulin and the remainder was albumin and β -globulin. The α -globulin present causes a marked turbidity. If this protein is not removed, the remaining fractions are frequently too turbid for electrophoresis.

The analyses of Fraction II + III of the plasma of control and burned rats are shown in Table III and the electrophoretic patterns are presented in Fig. 1. This fraction shows outstanding changes after the animal is burned. The percentage distribution then indicates that the α_2 and β components increase almost immediately (12 hours) and the γ -globulin concentration decreases rather markedly. These changes are reflected in the $\alpha_1:\gamma$ and $\beta:\gamma$ ratios.

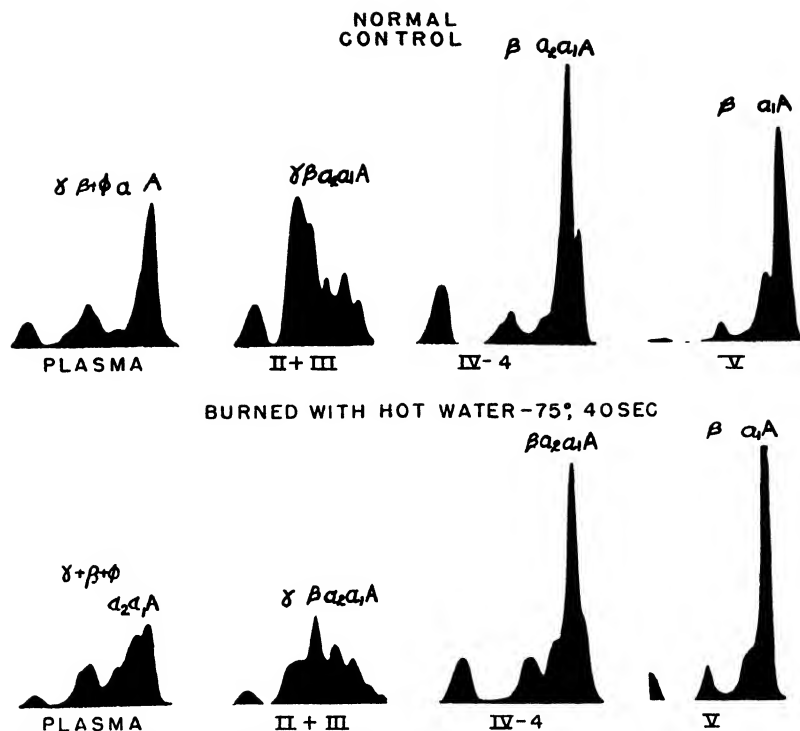


FIG. 1. Typical electrophoretic patterns for the plasma and plasma fractions of control and scalded rats.

TABLE IV
Electrophoretic Analyses of Fraction IV-4

Run No	Treatment	Days after injury	Albumin	α_1	α_2	β	γ
			per cent	per cent	per cent	per cent	per cent
44	Normal		13	56	9	14	8
46			21	53	9	10	5
49			11	59	12	17	
135	Burned		16	48	10	17	9
55		1	13	56	15	16	
75		1	19*	49	15	16	
56		2	10*	55	18	16	
53		3	5	53	23	14	3
57		3	8	46	26	15	4
74		3	11	37	24	27	
58		4	8	49	19	19	5
67		4	9	44	25	15	7
76		5	16	51	13	20	
77		5	18	40	14	19	
71		6	12	44	15	20	9

* Descending boundary.

The data for Fraction IV-4 (Table IV and Fig. 1) show no outstanding changes in distribution of its components after burning. The concen-

TABLE V
Electrophoretic Analyses of Fraction V

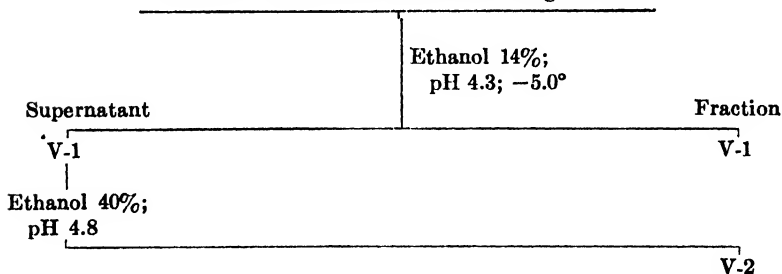
Run No.	Treatment	Days after injury	Albumin	α_1	α_2	β
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
44	Normal		78	14	3	5
46	"		69	18	4	7
47	"		68*	26		6
49	"		71*	19	5	5
78	"		66	23		11
101	"		70		20	10
55	Burned	1	68		21	11
56	"	2	74		15	11
66	"	3	62		27	11
74	"	3	65*	22		13
67	"	4	64	25		11
76	"	5	72	18		10
77	"	5	69	22		9

* Descending boundary.

DIAGRAM 2

Procedures for Removing Globulins from Fraction V

Fraction V dissolved in 10 times its weight of H₂O



Fraction V is easily soluble in H₂O. It is brought to a concentration of 14 per cent with 53.3 per cent ethanol and pH 4.3 with acetate buffer and the resulting precipitate is immediately removed by centrifuging. The ethanol concentration of Supernatant V-1 is then raised to 40 per cent with 95 per cent ethanol and the pH is raised to 4.8 with NaHCO₃. The precipitate is immediately removed by centrifuging.

tration of α_2 -globulin tends to increase somewhat during the first 3 days after burning.

No characteristic changes in the distribution of the Fraction V com-

ponents (Table V and Fig. 1) are seen after thermal injury. This fraction is characterized by the presence of appreciable amounts of α_1 - and β -globulins. These globulins may be removed by treating Fraction V according to the procedures outlined in Diagram 2. The patterns for

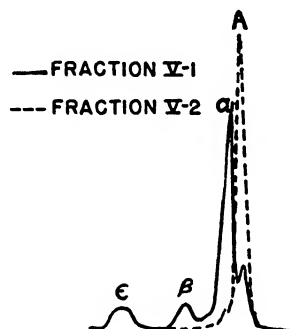


FIG. 2. Electrophoretic patterns after the subfractionation of Fraction V

TABLE VI
Nitrogen Distribution of Plasma Components

Fraction No.	Run No.	Treatment	Days after injury	N per 100 ml. plasma				
				Albumin	α_1	α_2	β	γ
				mg.	mg.	mg.	mg.	mg.
II + III	46	Normal		18	33	21	39	84
	74	Burned	3	3	39	34	49	38
	76	"	5	6	23	35	59	35
	77	"	5	13	37	45	80	41
IV-4	46	Normal		43	110	19	21	10
	55	Burned	1	36	153	41		44
	74	"	3	35	120	77		86
	76	"	5	47	151	39		59
	77	"	5	55	149	42		58
V	46	Normal		168	54		17	
	137	"		242	77		26	15
	55	Burned	1	56	17		10	
	74	"	3	123	41		25	
	76	"	5	172	43		24	
	77	"	5	139	44		18	

Fractions V-1 and V-2 are superimposed on each other by using the salt effect as a point of reference (Fig. 2). It is obvious that albumin that is almost electrophoretically pure is obtained by these methods.

Data for the nitrogen distribution of the components of the fractions are given in Table VI. Although the data are limited, they appear to

follow the general trend shown by the percentage distributions of the components in each fraction. The increase in the nitrogen concentration of α_2 - and β -globulins and the decrease in the γ -globulin are once more apparent in Fraction II + III after thermal injury. An increase in α_2 -, β -, and γ -globulins is observed in Fraction IV-4. The decrease in albumin in Fraction V is a characteristic finding for the rat and other species.

The mean values for the nitrogen contents of three fractions obtained from control and burned rats are shown in Table VII. The data for the burned rats include all values obtained during the first 5 days after burning. After injury significant decreases are seen for Fractions II + III and V.

TABLE VII
Yields of Fractions in Mg. of N Per 100 Ml. of Plasma

	Fraction II + III	Fraction IV-4 (a)	Fraction V (b)	$\frac{(b)}{(a)}$
Normal (8 rats)	195 \pm 5	233 \pm 13	317 \pm 15	1.36 \pm 0.14
Burned (18 ")	140* \pm 10	274 \pm 13	173* \pm 16	0.67* \pm 0.06

* Significantly different.

DISCUSSION

Electrophoretic patterns of normal rat plasma usually show two distinct boundaries which have considerable spreading. The two main components are classified as albumin and globulins. After fractionation of plasma, the boundaries of the components become clearly defined. These new boundaries are striking in the three fractions studied, particularly in Fraction II + III.

It would appear that the proteins of rat plasma exist as complexes which are easily split by the gentle treatment used in these fractionation procedures. The factors responsible for the close association of these components are not influenced by the electrical forces inherent in the electrophoretic method. It is planned to examine mixtures of these fractions to determine whether association of the various components will occur.

The increases in the α - and β -globulins and decreases of albumin and γ -globulin after thermal injury in the rat are similar to those obtained in the dog. The differences in the patterns for Fraction II + III of the control and injured animals are so characteristic that it is possible to classify them by visual inspection. These characteristic "injury" patterns of Fraction II + III are also observed after administration of turpentine and of adrenal cortical hormones.¹

¹ Gjessing, E. C., and Chanutin, A., unpublished data.

SUMMARY

A procedure is outlined for fractionating rat plasma into four portions by means of ethanol at low temperatures. Fractionation makes it possible to obtain reasonably accurate analyses for the distribution of the protein components.

Mobility data obtained from electrophoretic patterns of the components of rat plasma and its fractions are presented for the ascending and descending boundaries.

The electrophoretic analyses for the plasma and fractions of control and burned rats are given. Thermal injury gives rise to an increase in the concentrations of α - and β -globulins and a decrease in the γ -globulin and albumin.

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AN AUTOMATIC APPARATUS FOR THE DETERMINATION OF OXYGEN CONSUMPTION*

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Respirometers have been in use since the earliest days of physiology and an almost infinite variety of types have been described.¹ There exists at present, however, no simple flexible apparatus which will follow the changes in oxygen consumption over any considerable period of time without requiring laborious attendance. This lack greatly discourages the collection of any large mass of data on oxygen consumption or the routine use of oxygen consumption as a control or as a measure of gross metabolism and thus activity.

The apparatus here described was developed for a study of oxygen consumption in small mammals during 24 hour periods and possesses the following attributes: (1) it is almost completely automatic, requiring attention only at 6 to 12 hour intervals; (2) oxygen consumption is measured during a succession of periods which may be as short as 5 to 10 minutes or as long as desired; (3) the amount of oxygen consumed in each measured period may be quickly changed to accomodate different sized animals or to change the length of the period; (4) operation, calibration, and calculation are quick and simple; (5) except for a few simply constructed parts it may be assembled from standard equipment. Its practical utility has been demonstrated in studies which have included more than 75 runs on some ten species of mammals (Morrison and Pearson (6)²). By means of this apparatus the average basal metabolism of the white mouse (Swiss strain, average weight 21 gm., acclimated to 22-24° and measured at 28.5°) was determined to be 7.5 calories per gm. hour. This compares with values of 8.3 (Benedict and Fox (7)), 7.4 (Ebeling and Corey (8)), 7.5 (Davis and van Dyke (9)), 6.8 (Blank (10)), and 7.1 calories per gm. hour (Benedict and Lee (11)) reported by other authors. Although designed for small animals and applied to a particular closed circuit system, the automatic and recording features are adaptable to any size of apparatus and to other types, including those modified for measurement of carbon dioxide production at longer intervals.

* This study has been assisted by a grant from the Ella Sachs Plotz Foundation.

¹ For descriptions of various respirometers see, for example, Krogh (1), Benedict (2-4), and Brody (5).

² Morrison, P. R., unpublished work. Pearson, O. P., *Ecology*, **28**, 127 (1947).

Principle

A closed system is maintained at constant pressure by means of a delicately balanced spirometer which descends as oxygen is consumed. The carbon dioxide produced is absorbed on soda lime in the chamber itself and, for small animals, convection and diffusion provide adequate circulation. The excursion of the spirometer is limited by two pairs of electrical contacts. These 'act through a relay system' to control a gas-tight solenoid valve which is interposed between the spirometer and a reservoir of oxygen and which allows automatic refilling of the spirometer.

The sequence of events in each cycle is as follows: (1) oxygen is consumed, and the spirometer descends; (2) the No. 1 (lower) contacts "make," and the solenoid valve opens; (3) oxygen flows, and the spirometer rises; (4) the No. 2 (upper) contacts "make," and the solenoid valve closes; (5) oxygen is consumed, the spirometer descends, and the No. 2 contacts "break." The time interval between the make of the No. 1 contacts and the break of the No. 2 pair is recorded on a moving paper strip. An electric timer records appropriate intervals on the same strip. The rate of oxygen consumption for any period is computed from the volume of the spirometer between contacts and the time taken to consume this volume; *i.e.*, from the distance between marks on the record. With successive values a continuous curve describing the rate of oxygen consumption as a function of time may be plotted.

The success of this apparatus derives in part from the fact that time, a quantity which is easily measured with great precision, is the dependent variable and the volume consumed is fixed. This principle has been applied by Benedict (2, 3) to non-automatic types of respirometers. Lewis and Luck (12) have described an apparatus with a similar basic principle in which an automatic siphon arrangement was employed to meter successive volumes of oxygen. Werthessen (13) has also described a quite elaborate automatic apparatus for this purpose which supplied oxygen electrolytically and embodied a recording ammeter.

Description

A schematic plan of the apparatus is shown in Fig. 1. The chamber and all removable connections were submerged in a large thermostated water bath to prevent leaks and fluctuations in temperature. Under this positive water pressure any leak is immediately manifest by the presence of water inside the system. The spirometer and its bath are mounted above the large bath and may be enclosed with it for protection against mechanical injury and temperature changes. Alternatively the spirometer bath may be mounted directly in the large thermostated bath, although temperature variation here is much less important than in the chamber itself. A change

of 3° is needed to introduce a 1 per cent error, while a change of only $0.1-0.2^{\circ}$ in the temperature of the much larger chamber during a single measuring period will introduce a like error.

A glass-lined brass chamber of 1 liter volume was generally used, but the dome-shaped top of a vacuum desiccator made a convenient and available substitute. The glass cover or base was sealed to the chamber with petroleum, which was quite satisfactory at these temperatures (up to 30°) and was much easier to remove and clean than stiffer lubricants. In addition to the spirometer connection another outlet was provided for sampling.

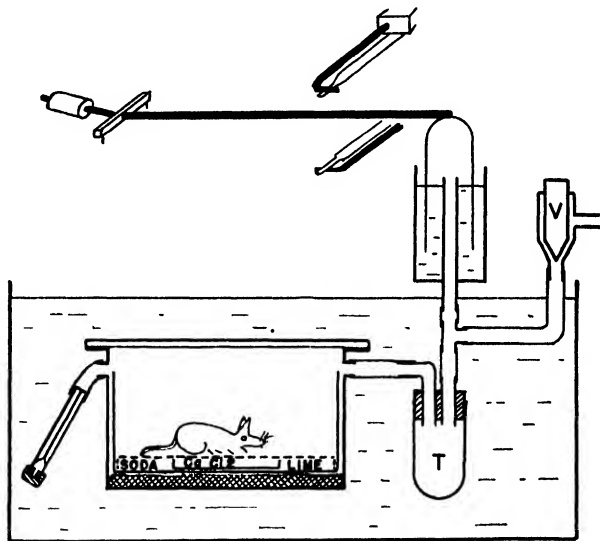


FIG. 1. A schematic diagram of the apparatus. The cross-hatched area represents a layer of lead to make the closed chamber heavier than water. The trap (*T*) is grossly out of proportion, having an actual volume of only 10 to 20 cc. Oxygen enters through value *V*.

This was conveniently done through a vaccine cap with a hypodermic needle. The cap was connected to the outlet through a length of capillary tubing and lay near the bath surface so that samples could be taken with a minimum of disturbance and without wetting the sampling syringe. The bottom of the chamber was divided into two concentric sections, holding layers of soda lime and wet calcium chloride respectively. The latter functioned to keep the humidity within reasonable limits. If such provision is not made, water will condense and wet the animal's fur and make it very uncomfortable. Saturated calcium chloride is in equilibrium with a relative humidity of 31 per cent at 25° (Koehler (14)) but the effective

humidity in the chamber is undoubtedly higher. A small trap was interposed between the chamber and the spirometer to catch any moisture which might condense in the connecting tubes or become lodged there through injudicious manipulation of the spirometer. The animal was separated from the absorbents by a layer of screen which allowed free circulation. Dr. P. F. Scholander has pointed out that under these conditions ammonia may be formed from urine in the soda lime. While the soda lime might be shielded, this would reduce the effective circulation in the chamber. It would appear preferable to meet this possibility by wetting the calcium chloride with dilute acid or buffer instead of water and thus absorb any ammonia so formed.

Details of the spirometer mounting and its pivots³ and of the electrical contacts are shown in Fig. 2. Aluminum cigar containers were employed as spirometers³ in measuring volumes smaller than 15 cc. In this situation a delicate and yet positive action was required, together with a mounting which produced no horizontal displacement on rotation (note Fig. 2, *B*). A ring of wire solder was cemented to the lower edge of the spirometer to insure vertical hanging and solutions of detergents (Nacconol, Turgitol, Xepherin) were used in the spirometer bath to lower the surface tension. Larger spirometers (30 to 80 cc.) were constructed of sheet aluminum. It was necessary to lacquer these spirometers to prevent electrolysis by stray currents.

The basic circuit design used in controlling the apparatus is shown in Fig. 3. The coil of a ratchet relay may be energized through a primary relay by closing either the No. 1 (bottom) or the No. 2 (top) pair of contacts. Successive closing of this circuit alternately closes and opens the relay contacts and thus the solenoid valve. The signal is also actuated through the primary relay. This is important, since time must be measured not merely during filling but until the No. 2 contacts open again. This frees the system from errors due to delayed closing of the solenoid valve; such lags have sometimes been observed in practice.

In the circuit shown in Fig. 4 this basic design was modified to permit the use of equipment already on hand. The ratchet relay was replaced by two simple relays, *R1* and *R2*, whose coils and contacts were respectively arranged in series. Once actuated through the No. 1 contacts, the circuit was self-energizing and remained closed until broken at some other point. This was accomplished through another relay, *R3*, on closing the No. 2 contacts; an appropriate shunt might also be used. The ratchet relay may also be replaced by a mechanical latch, electrical reset type relay couple. In this case the latch coil is connected through the No. 1 contacts and the release coil through the No. 2 contacts.

³ I am indebted to Dr. P. F. Scholander for advice in these mechanical details and to Dr. A. C. Redfield for suggesting this convenient source of tiny spirometers.

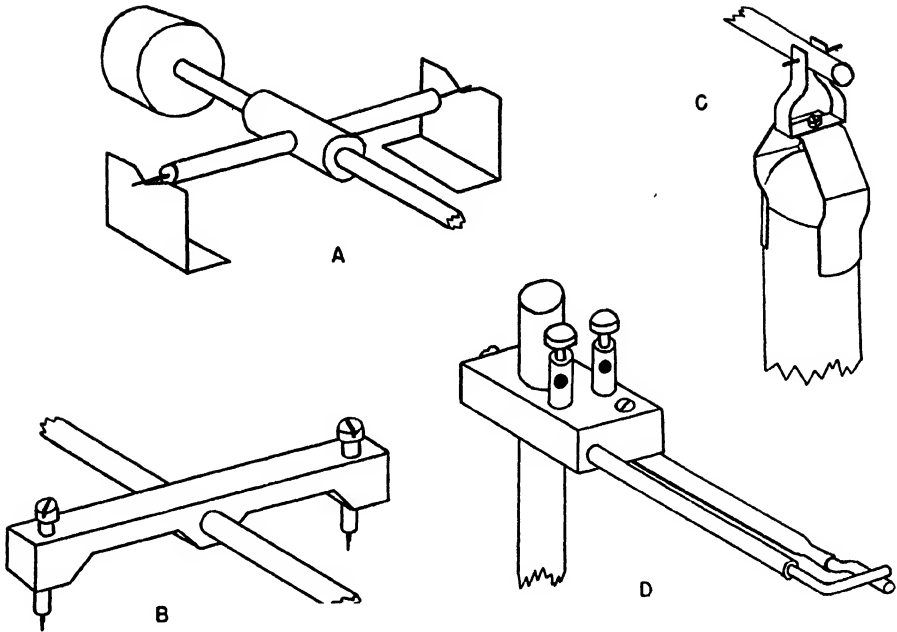


FIG. 2. Details of construction. *A* and *B*, alternative pivoting arrangements for the spirometer arm. The sections were threaded together and the counterweight threaded on for precise adjustment. The long section supporting the spirometer was of aluminum rod or tube; other parts were of brass or bakelite. The actual pivots in both cases were plated steel phonograph needles which were fitted closely, but not fixed, so that replacement was easy. *C*, spirometer mounting; upper section of phosphor bronze to facilitate removal; lower section of aluminum cemented to the spirometer. *D*, upper pair of contacts; the binding post and contacts were mounted on a bakelite block which could be fixed at different positions on the vertical supporting rod. The movable contact of phosphor bronze was provided with an adjustment screw. The contact tips should be of corrosion-resistant metal, such as silver or platinum.

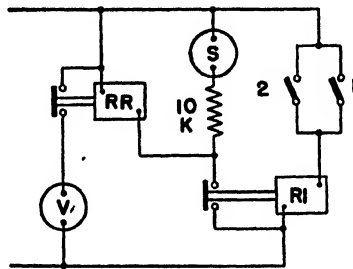


FIG. 3. Basic control circuit. *RR*, ratchet relay in which successive coil impulses open and close the relay contacts; *V*, solenoid valve; *S*, signal. The relay, *RI*, should be moderately sensitive to reduce sparking and corrosion at the contacts, Nos. 1 and 2.

Oxygen was supplied from a large reserve spirometer under a very low pressure (1 to 3 cm. of water) rather than directly through a reducing valve. Under these conditions leakage through the closed valve was never observed. At first a low voltage signal pen with its auxiliary circuit was used (Fig. 4), but this was subsequently eliminated by the use of direct recording paper⁴ which utilized 110 volts A.C. through a 10,000 ohm resistor. This elegant recording procedure not only simplifies the circuit (Fig. 3) but also facilitates the use of duplicate set-ups and the simultaneous recording of other information, such as activity, feeding, etc.

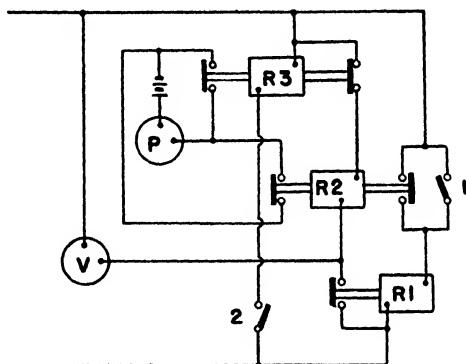


FIG. 4. Modified control circuit, with simple relays, $R1$, $R2$, and $R3$, and a low voltage signal pen, P ; other symbols as in Fig 3.

Procedure

Operation—After the animal was inserted in the chamber, a period of about an hour was allowed for the chamber and the animal to come into equilibrium. During this period the temperature and the carbon dioxide concentration approached their equilibrium values. Fig. 5 shows the apparent oxygen consumption during this period as taken from the record of a flying squirrel (*Glaucomys*).

During the run samples of air from the chamber may be removed for analysis in the Haldane apparatus or more conveniently by the method of Scholander (15) to insure that the soda lime is absorbing properly and that oxygen is in sufficient concentration. Because water was used in the spirometer baths to avoid any odor which might disturb the sensitive animals under investigation, the oxygen delivered into the chamber was not pure. This diffusion of nitrogen through the water baths was added

⁴ Teledeltos recording paper (type L), used in facsimile recording instruments, is manufactured by the Western Union Telegraph Company, 60 Hudson Street, New York.

to any original impurities in the gas as supplied. Although this does not affect the calibration, over a period of hours it will lower the oxygen tension in the chamber. This may be simply met by periodic withdrawals (one to three per day) of air which is then automatically replaced by oxygen from the spirometer. The withdrawals for analysis often suffice to maintain the oxygen concentration. In practice, the volumes added have corresponded to an entering oxygen concentration of 98 to 99 per cent.

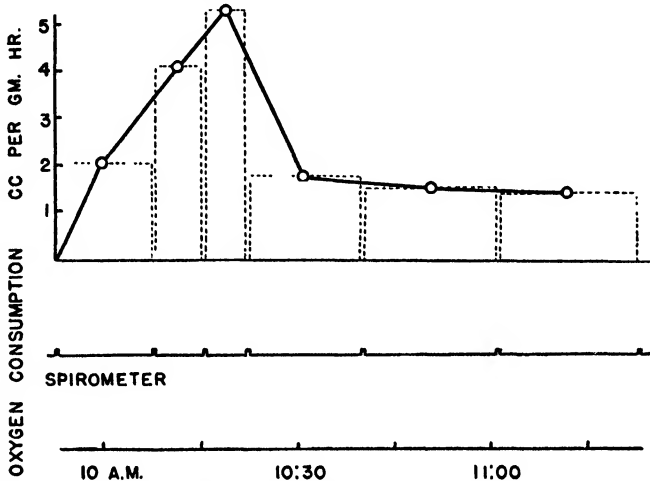


FIG. 5. A record (below) and its conversion into oxygen consumption. Flying squirrel (*Glaucomys*), December 19. This record immediately follows the insertion of the animal into the chamber and shows a period of very intense activity followed by sleep. The broken rectangles represent consumption of one spirometer of oxygen each. Note the initial lag in the measured oxygen consumption as the system attains equilibrium. This is an extreme case and, in practice, the 1st hour is not used.

Calibration—The amount of gas delivered by the spirometer was determined by slowly withdrawing air through the sampling tube into a syringe of appropriate size. With the spirometer full at the start, the syringe positions corresponding to the break of the No. 2 (upper) contacts and the make of the No. 1 (lower) contacts are noted, the difference defining the volume. The syringe itself was calibrated by delivery of water or mercury. In converting this volume into amounts of oxygen (expressed as dry gas at 0° and 760 mm. of Hg pressure), the temperature used is that of the calibrating syringe, not that of the chamber or spirometer, and the entering oxygen is considered to be saturated with water vapor. A spirometer calibration is shown in Fig. 6, which illustrates the delicacy of the tiny spirometers. During the period of free fall of the spirometer no pressure change could be observed (*i.e.* less than 0.2 mm. of water). After

the arm strikes the lower contacts, the pressure drops slightly, although the larger part of the additional volume refers to the continued fall of the spirometer until the contacts made. This pressure difference introduces into the calibration a small error which is proportional to the chamber volume and to the square of the relative change in pressure ($v(\Delta P/P)^2$). This error is completely negligible here, amounting to less than 0.1 c.mm. With the chamber not attached, the effective volume of the spirometer was 0.1 to 0.2 cc. lower and this difference is again a function of the

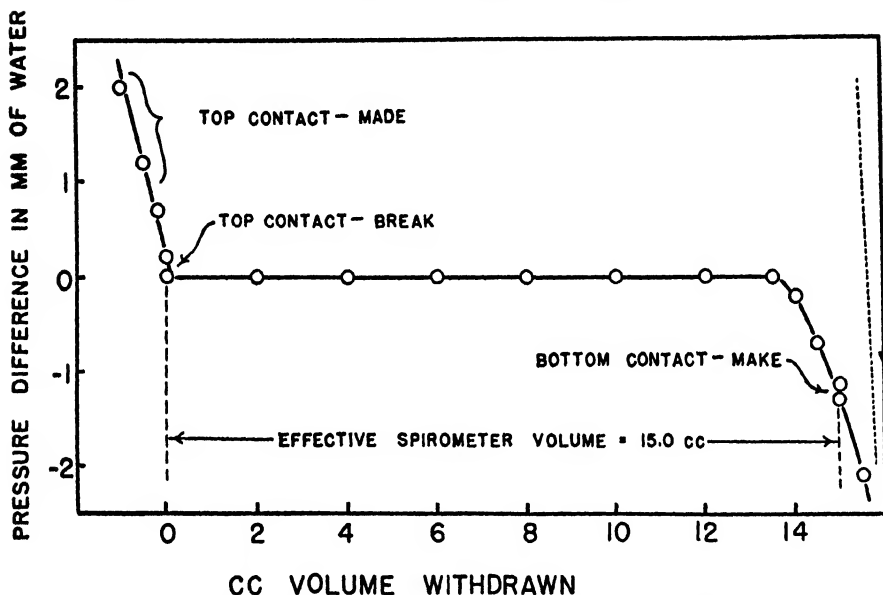


FIG. 6. Calibration of the spirometer, showing the pressure differential in the chamber as a function of the spirometer position. The broken curve at the left with a slope of 1 cc. per cm. of water shows the volume referable to a change in pressure in a 1 liter chamber.

chamber volume and the relative pressure change ($v(\Delta P/P)$). Successive calibrations never differed by more than ± 0.1 cc. in 10 cc. and usually gave exactly the same value. The spirometer was calibrated at the beginning and end of each experiment and routinely showed the same value, even after 50 to 100 operating cycles.

Calculation—A section of a record and its conversion into oxygen consumption were shown in Fig. 5. The time signal showed that the recording paper was moving at uniform speed. Each of the dotted rectangles corresponds to the utilization of one spirometer of oxygen and all are thus of equal area. The small distances between the rectangles corresponding to time of filling the spirometer are a little exaggerated in Fig. 5 and usually

amounted to about half a minute. The length of the measured period is inversely proportional to the rate of oxygen consumption. This is an advantage in that measurements will be more frequent while the animal is active, at which time more rapid changes in metabolism are expected. Since the various conditions of the experiment, the speed of the record, the weight of the animal and the volume of the spirometer, may be assembled into a single constant, f , the calculation of even a large number of periods is not too laborious. Further, the measurement on the record, a simple distance, is also simple and is only slightly dependent on subjective judgment. If the oxygen consumption in any single period is given by $M = f/(\text{mm.})$, then the average rate over n periods will be, $M_{\text{ave.}} = nf/2(\text{mm.})$. In converting these amounts into units of energy, the advantage of measuring oxygen consumption rather than carbon dioxide production is clear, since the caloric equivalence of the oxygen consumed is much less dependent on the respiratory quotient, varying within less than ± 1.5 per cent as the latter (protein-free) varies between 0.77 and 0.88.

DISCUSSION

In designing this particular apparatus attention was given to the well being and comfort of the animal, and several features, notably the generous sized chamber and the absence of a pump or fan, were embodied despite a concomitant reduction in sensitivity. This is in contrast to the more usual procedure of defining the conditions of measurement with little regard for the animal. However, in view of the close dependence of the metabolism on the degree of activity or repose, it is not unreasonable to regard the comfort of the animal as of comparable importance with the physical characteristics of the system. When this is done, it is necessary to evaluate critically the influence of design on the sensitivity and accuracy of the system so that the proper compromise between these two factors may be reached. In defining the proper limits of accuracy the fact that the standard deviation of metabolic measurements is of the order of 10 per cent should be borne in mind. In the apparatus described in detail here the standard deviation of minimum or basal values referable to experimental errors is estimated as 2 to 4 per cent.

One important potential source of error results from changes in the carbon dioxide concentration following a change in the metabolic rate. This is a consequence of the dependence of the rate of carbon dioxide absorption on the carbon dioxide concentration, and in a system in which a fraction of the volume is actively circulated through a carbon dioxide absorber at a rate, r , the following relation will hold,

$$r \left(\frac{\% \text{ CO}_2}{100} \right) = (\text{cc. CO}_2 \text{ per min.})$$

Because of these changes in the carbon dioxide concentration the measured oxygen consumption (M) will lag behind the actual oxygen consumption (A). These relations may be calculated thus, for $M = A = 0$ at $t = 0$; and R.Q. = 1.0:

Since the rate of carbon dioxide absorption is the measured rate of oxygen consumption,

$$\frac{M}{r} = \frac{\%CO_2}{100}$$

But since the carbon dioxide concentration in the chamber represents the accumulated difference between production and absorption,

$$\frac{\%CO_2}{100} = \int (A - M) \frac{dt}{v}$$

where v is the volume of the chamber.

If these are combined,

$$dM = \frac{r}{v} (A - M) dt$$

and

$$\frac{A - M}{A} = e^{-(r/v)t}$$

or

$$\frac{M}{A} = 1 - e^{-(r/v)t}$$

This function which is of general application to closed circuit systems has been plotted in Fig. 7 for values of r/v (the *fractional circulation rate*) ranging from 0.05 to 1.0 per minute. The area above each curve represents the difference between production and absorption or the increased amount of carbon dioxide in the chamber. Also shown in Fig. 7 are the average values which would be measured in finite periods of 5, 10, and 20 minutes.

In practice, this function will apply to changes in the rate of oxygen consumption from one level to another, thus, $A \rightarrow A + \Delta A$ (with $A = M \neq 0$ at $t = 0$), and it will describe $\Delta M / \Delta A$ as a function of time. Negative changes or decreases in the rate of oxygen consumption will behave in the same manner as positive ones. Sudden changes in the oxygen consumption will usually be caused by muscular activity and characterized by a respiratory quotient of 1.0, but even with different values there is not too great deviation from this function.⁵

⁵ When the R.Q. is lower than 1.0, one fraction $(1 - \text{R.Q.})$ of the change in oxygen consumption will be seen immediately, while the remaining fraction ($= \text{R.Q.}$) follows the function above. For example, with an R.Q. of 0.80, one-fifth of the change would be manifested at once and four-fifths would follow the relations in Fig. 7.

It will be seen that there is a definite relation between the fractional circulation rate and the shortness of period which it is profitable to employ. Thus, even at the high rate of 1.0 per minute (*i.e.* the volume of the chamber circulated each minute) there is little advantage in using periods shorter than 5 minutes, and at rates lower than 0.25 per minute 10 minute periods appear adequate to follow any changes in the system.

Changes in the temperature of the chamber will cause a similar lag. If the heat loss from the chamber is considered to follow Newton's law of

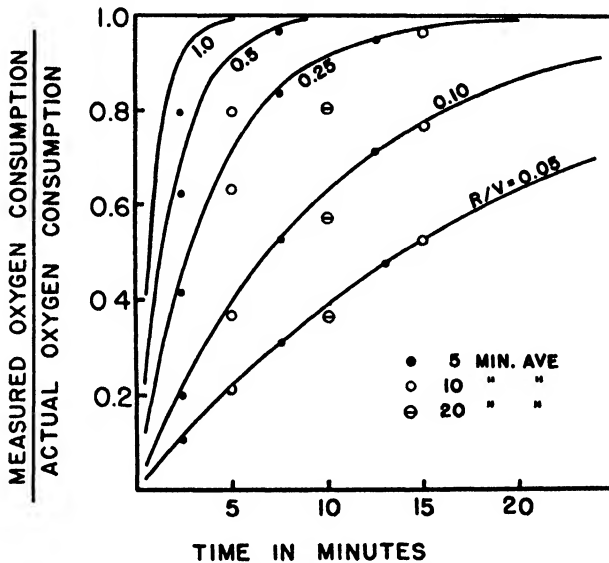


FIG. 7. The relation between the measured oxygen consumption (M) and the actual oxygen consumption (A) as a function of time at different fractional circulation rates. The curves are computed from the relation, $M/A = 1 - e^{-(r/v)t}$. The points represent average values for successive finite periods and each belongs to the curve directly above.

cooling with the rate of heat transfer proportional to the difference in average temperature inside and outside the chamber, then equations of the same form may be set up to describe the change in temperature with time following a change in heat production. However, a different rate constant will apply and the volume term will be replaced by the heat capacity of the system. The temperature error so introduced will be $v\Delta T/T + 273$, where ΔT is the change in temperature during the measured period.

In the apparatus described in detail here circulation was not effected actively, but passively through convection. It seems clear, however, that

under a given set of conditions the rate of absorption should again be proportional to the carbon dioxide concentration. By analogy to the apparatus with active circulation, we can calculate an effective circulation rate, r' , from the measured values of oxygen consumption and of carbon dioxide concentration thus,

$$r' = (\text{cc. CO}_2 \text{ per min.}) / \left(\frac{\% \text{CO}_2}{100} \right) = (\text{cc. O}_2 \text{ per min.}) (\text{R.Q.}) / \left(\frac{\% \text{CO}_2}{100} \right)$$

Such experimental values are shown in Fig. 8, together with contours representing different circulation rates. On this basis values for r' in

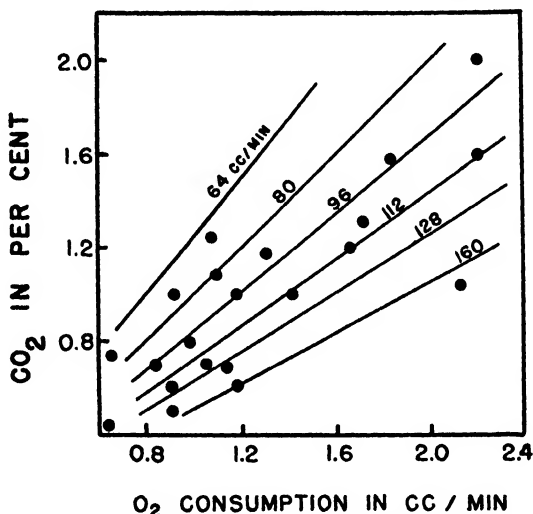


FIG. 8. The carbon dioxide content of the chamber as a function of the rate of oxygen consumption. The points represent individual measured values. The contours show the effective circulation rate, $r' = (\text{cc. of O}_2 \text{ per minute}) (\text{R.Q.}) / (\text{per cent CO}_2)$, calculated for an R.Q. of 0.80.

this apparatus ranged from 70 to 170 per minute and averaged 110. This corresponds to a fractional circulation rate of about 0.15. The apparent rise in oxygen consumption during a period of relatively constant activity, as is seen in Fig. 6, reflects these changes in carbon dioxide and temperature. The error attributable to temperature (1–2° per cc. of O₂ per minute) is about half that due to the accumulation of carbon dioxide and is in the same direction.

In certain species (*e.g.* the shrew, *Blarina*, and the bats, *Myotis* and *Eptesicus*, in which periods of intense activity alternate with longer periods of almost complete inactivity) the fall in rate of oxygen consumption after a period of activity can be analyzed and compared to the theoretical curves

in Fig. 7. This provides an independent estimate of the fractional circulation rate; values so obtained ranged from 0.08 to 0.15 per minute, in general agreement with those estimated from the carbon dioxide concentration (Fig. 8).

The measured oxygen consumption in any given period, then, will differ from the actual rate of consumption. The size of this difference will be related to the levels in the previous periods and particularly in the one immediately preceding. In short peaks of activity considerable error can be introduced; in measuring periods of minimum activity, as for a basal metabolic rate, when the period is usually preceded by others of comparable magnitude, these errors will be small; and in measuring the average rate over a number of periods they will be negligible. These lag or accumulation errors will be added, of course, to any other errors such as calibration errors or those resulting from changes in the atmospheric pressure.

SUMMARY

A closed circuit system for the automatic measurement of the rate of oxygen consumption is described. The time required to empty a small, accurately calibrated spirometer is recorded kymographically and the spirometer is refilled automatically through an electrically controlled solenoid valve. Some errors inherent in closed circuit systems in general and their quantitative dependence on the physical characteristics of the system are discussed.

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AN IMPROVED METHOD FOR THE DETERMINATION OF THIOURACIL*

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Several adaptations of Grote's (1) reagent for the determination of compounds containing the thiourylene group have been applied to the determination of thiouracil (2-4) and thiourca (5-8). All of these workers report disadvantages for the methods when used in routine determinations. The necessity for running standard samples with each set of unknowns is time-consuming and inconvenient. In general, the disadvantages of the above methods are (1) instability of the stock reagent, (2) inconvenient adjustment of the reaction, (3) inability to adjust the samples to a uniform reaction, (4) excessive time required to develop maximum color intensities, and (5) preparation of standards for each set of determinations.

Except for Williams *et al.* (2), all workers have reported an unstable color reagent which necessitates not only the preparation of a fresh reagent, but also the inclusion of standards for each group of determinations. His method has the disadvantage, however, of a 12 hour enzyme digestion and a tedious adjustment of the reaction with strong base. To avoid the occurrence of cloudiness observed by Williams *et al.* after addition of the reagent, we have followed the observation of Grote (1) that a more stable color is produced by allowing the color reaction to occur near neutrality. We have accomplished this by the use of a phosphate buffering system in the diluted reagent, having a buffering capacity sufficient to give an identical pH in all unknown or standard samples analyzed. It has been demonstrated by Chesley (8) that the pH is a critical factor in the development of uniform color intensities. By a process of aging the stock color reagent, we have been able to show reproducible results with different lots of color reagent, as well as with a single lot of reagent used over a long period of time. The time required for maximum color development has been greatly reduced by allowing the color reaction to develop at 50°.

Standardization of the above conditions permits the determination of standard absorption curves that are reproducible within the limits of experimental error. The elimination of the necessity for establishing a standard curve with each set of determinations is a distinct advantage.

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Reagents—Except for minor modifications, the reagents used in this investigation were identical with those of Chesley (8). Plasma proteins were precipitated by $\frac{2}{3}$ N tungstic acid prepared immediately before use by mixing equal parts of 10 per cent sodium tungstate and $\frac{2}{3}$ N sulfuric acid.

To prepare the color reagent, 10 ml. of a 5 per cent solution of sodium nitroferricyanide and 5 ml. of 10 per cent hydroxylamine hydrochloride are pipetted into a 50 ml. Erlenmeyer flask, gently mixed, and allowed to stand for 2 minutes. Then add 10 ml. of 10 per cent sodium bicarbonate, mix gently, and in 10 minutes add 0.11 ml. of bromine. During this interval the reagent will turn from a light brown to a dark brown color, accompanied by the evolution of gas. After 10 minutes 5 ml. of 2 per cent phenol are added with gentle mixing. The reagent is now allowed to stand at room temperature for 5 to 12 hours and is then placed in the refrigerator at approximately 5°. The solution will assume a green color after this aging period, and if kept under refrigeration will reproduce reliable standard curves up to 75 days. This observation was found to be a distinct advantage, as it allows the preparation of double or triple lots of reagent at one time. Filtration of this stock reagent will not alter its reproducibility. A stock reagent may be prepared by varying the time intervals reported. However, the importance of accurately controlling these time intervals in order to produce a consistent and reproducible reagent has been pointed out by Chesley (8). The stock reagent may be used before aging, but it was found that in such cases a standard curve must always be plotted in order to insure accurate values.

For use in color production the stock reagent is diluted 1:20 with phosphate buffer. This buffer is composed of equal parts of 0.2 M disodium phosphate and 0.2 M monopotassium phosphate mixed just before use. In view of the fact that color intensity is a function of hydrogen ion concentration, high buffering capacity is desirable. This reagent was found adequate for adjusting all samples and standards to pH 6.8. It is well to mention in this connection that the pH at which the components of the stock reagent react is critical for subsequent color development and that the distilled water used in making all reagents should approximate neutrality.

A convenient apparatus for measuring the bromine used in preparing the color reagent is a micro burette or a suitably graduated pipette with a stop-cock fused on the delivery end. A vacuum may be applied to the burette with a rubber bulb or large hypodermic syringe and the bromine drawn from the stock bottle through a piece of small glass tubing which is temporarily fastened to the delivery end of the burette by a rubber coupling.

Reproducibility of Method with Thiouracil Standard Solutions—Twelve sets of reagents were used to establish their constancy in producing identical results.

Table I contains data collected on three individual preparations of stock color reagent that were aged at various time intervals of 5 hours to 75 days. Any stock color reagent aged in excess of 24 hours was maintained at refrigerator temperatures. Transmission values were measured at 660 $m\mu$ and the extinction values ($\log I_0/I$) were calculated and plotted against the respective thiouracil concentrations.

The data in Table I show that the average deviation from the mean extinction values lies within the limits of 2.91 and 1.04 per cent, with the greater variation existing at the lower thiouracil concentrations.

TABLE I
*Reproducibility of Stock Color Reagent**

Reagent No.	Age	Extinction values				
		0.6 mg. per cent	1.2 mg. per cent	1.8 mg. per cent	2.4 mg. per cent	3.0 mg. per cent
I	48 hrs.	0.199	0.390	0.585	0.770	0.959
"	96 "	0.194	0.395	0.585	0.770	0.939
"	10 days	0.187	0.382	0.561	0.745	0.912
II	5 hrs.	0.188	0.380	0.565	0.755	0.930
"	100 "	0.210	0.395	0.575	0.755	0.930
III	48 "	0.196	0.395	0.585	0.770	0.947
"	75 days	0.196	0.393	0.580	0.765	0.949
Mean		0.196	0.390	0.576	0.761	0.938
Average deviation, %		2.91	1.32	1.44	1.11	1.04

* These values were obtained with a Coleman universal spectrophotometer. A wave-length of 660 $m\mu$ and a light path of 4 cm. were used.

Fig. 1 shows a curve representing the mean values of the seven individual runs recorded in Table I. This standard curve may be used for routine determinations with the color reagent described. By including two or more standard solutions within the range of these concentrations, the suitability of any color reagent may be conveniently checked.

Procedure

Preparation of Serum or Plasma—Equal volumes of serum or plasma and $\frac{2}{3}$ N tungstic acid are placed in a centrifuge tube and thoroughly mixed. The mixture is centrifuged to aid filtration and the supernatant liquid solution filtered. The filtrate is ready for color development as described below. When the 6 ml. Evelyn macro tubes or the round cuvettes in the Coleman universal spectrophotometer are used, 4 ml. of plasma or serum are sufficient for single determinations.

Preparation of Urine—Urine is diluted with distilled water to a concen-

tration giving color intensities in the range of optimal accuracy for the instrument used. Urine dilution of 1:10 gave satisfactory results when effective blood plasma levels were maintained.

Color Development—The filtrate or diluted urine is pipetted into colorimeter tubes and warmed to 50° in a water bath before the diluted color reagent is added. 3 ml. of color reagent, also warmed, are added to 3 ml. of the sample and transmission readings are taken at 5 minute intervals against a blank of distilled water and color reagent. At this temperature maximum color intensities are developed in from 5 to 15 minutes with the usual range

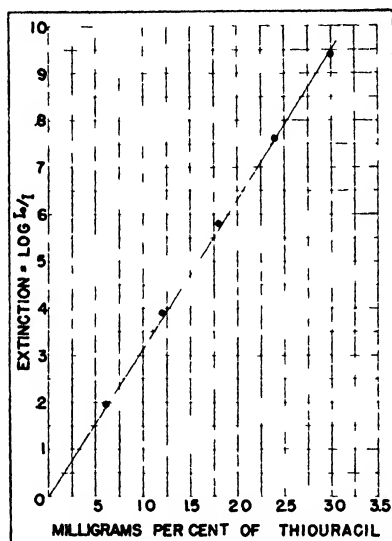


FIG. 1. Mean extinction values of seven individual runs with standard solutions. Determinations were made on three different reagents aged at intervals reported in Table I.

of concentrations. The color may be developed at room temperature, but it occurs at much slower rates, and greater variations also occur in the length of time required for different concentrations to reach their maximum intensities.

The intensity of color developed is determined with a photoelectric colorimeter with a 660 μ light setting or a filter having maximum transmission at 660 μ . Fig. 2 illustrates that transmission values taken at 660 μ and at pH 6.8 are satisfactory.

To check reproducibility, two or more concentrations of thiouracil standard solution may be included in each series of samples and their extinction values checked with the standard graph.

Calculations—The concentration of thiouracil may be calculated by converting the extinction value for the unknown into mg. per cent from the standard graph, or, if more convenient, by the use of the k value according to the formula $C = E/k$, where C = concentration of thiouracil, E = extinction or I_0/I , and $k = E/C$. In both instances the proper correction for dilution of the original material must be made.

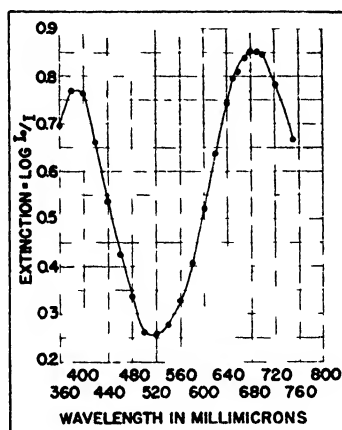


FIG 2 Absorption spectrum of Grote's reagent in a thiouracil solution at pH 6.8. Measurements were made with a Coleman universal spectrophotometer with a 4 cm cuvette

TABLE II
Effect of Storage on Thiouracil in Plasma and Urine

Interval days	Plasma		Plasma filtrate		Urine	
	mg per cent	per cent loss	mg. per cent	per cent loss	mg per cent	per cent loss
0	2.95		2.95		21.75	
1	2.85	3.4	2.95		21.75	
2	2.86	3.1	2.94	0.3	21.50	1.1
4	2.87	2.7	2.93	0.7	21.25	2.3
6	2.55	13.6	2.75	6.8	20.90	3.9
7	2.55	13.6	2.85	3.4	20.90	3.9

Decomposition of Plasma, Urine, and Plasma Filtrates—Stability of thiouracil in plasma, urine, and plasma filtrates held at refrigerator temperature was studied. Plasma and tungstic acid filtrates prepared from the same plasma sample were stored in the refrigerator for varying periods of time. The urine sample was filtered and diluted just prior to the determination.

The results shown in Table II indicate that plasma, plasma filtrates, or

urine may be held at refrigerator temperature for periods up to 4 days without excessive loss of thiouracil. Greater loss occurred in the plasma than in the plasma filtrate up to 4 days and also after a week's storage.

DISCUSSION

The use of an aged color reagent which produces an intensity of color with thiouracil standards that is reproducible for comparatively long periods of time is reported and a rapid and accurate method for the determination of thiouracil in blood and urine described.

The use of this improved stock reagent avoids the plotting of standard curves for each determination and eliminates the time-consuming preparation of frequent lots of color reagent. While noticeable changes will appear in both the stock and diluted color reagents over a period of time, reproducible color intensities are secured with standard solutions of thiouracil. It is advisable to filter the stock reagent before diluting with the buffer.

By the use of this stable color reagent, larger quantities of the stock reagent may be prepared at one time, thus permitting use of the same stock reagent through a continued study. Comparison of standard solutions at intervals will verify its original color development. Our results indicate that this aged stock reagent will give reproducible results for several weeks.

The effect of pH on the intensity of color produced with reagents of this type makes it extremely important to have sufficient buffering capacity to insure identical reactions in all samples. This is accomplished by the use of 0.2 M phosphate buffer at pH 6.8.

Filtrates of normal bovine and goat plasma showed light absorption equivalent to 0.3 mg. per cent or less of thiouracil when compared with a blank of distilled water. The small error due to this non-specific absorption can be minimized by use of a normal plasma filtrate blank.

SUMMARY

A stock color reagent for thiouracil determinations giving reproducible results over a considerable period of time is reported.

Maximum buffering capacity of the diluted color reagent is secured by the use of 0.2 M phosphate buffer.

Preparation of a standard curve for any laboratory photoelectric colorimeter is feasible.

Development of maximum color intensities in from 5 to 15 minutes at 50° is recommended.

For all practical purposes the loss of thiouracil in plasma filtrates and urine stored for 4 days at refrigerator temperature is of a negligible order.

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GROWTH INHIBITION OF BACTERIA BY SYNTHETIC PTERINS

I. STUDIES WITH *STREPTOCOCCUS FAECALIS*, *LACTOBACILLUS CASEI*, AND *LACTOBACILLUS ARABINOSUS**

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Woods (1) has shown that *p*-aminobenzoic acid (PABA) antagonizes the sulfanilamide inhibition of bacterial growth. Lampen and Jones (2) found that pteroylglutamic acid (folic acid) and related compounds are also active antagonists of sulfonamide inhibition of certain microorganisms. They concluded from their results that the inhibition of bacterial growth by sulfonamides was caused by interference in the synthesis of folic acid at the PABA site in the folic acid molecule.

The studies reported in this paper demonstrate that growth inhibition of bacteria is likewise obtained with pterins¹ related structurally to the pteridine portion of the folic acid molecule. The studies include work with three organisms, *Streptococcus faecalis*, *Lactobacillus casei*, and *Lactobacillus arabinosus*. *Streptococcus faecalis* and *Lactobacillus casei* require folic acid in the medium and *Lactobacillus arabinosus* synthesizes its own requirements of this vitamin. Studies with other microorganisms and biological results obtained with chicks will be reported in later publications.

EXPERIMENTAL

Cultures and Media—Cultures of *Streptococcus faecalis* (*S. lactis* R), *Lactobacillus casei*, and *Lactobacillus arabinosus* were obtained from the American Type Culture Collection. They were maintained as agar slabs in liver, glucose, yeast, and tryptone agar, the composition of which is described by Nymon, Gunsalus, and Gortner (3). Weekly transfers were

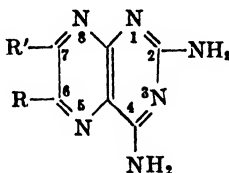
* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D. C., and was aided by a grant to Cornell University by the Nutrition Foundation, Inc., New York. The work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry.

¹ The synthesis of these compounds is being reported elsewhere by M. F. Mallette, E. C. Taylor, Jr., and C. K. Cain, of the Department of Chemistry, Cornell University, Ithaca, New York.

made through a broth of the same composition, except for the omission of agar.

The medium used for *Streptococcus faecalis* was that of Luckey, Briggs, and Elvehjem (4), modified to include 200 millimicrograms of pyridoxamine per tube. For *Lactobacillus casei* the medium of Teply and Elvehjem (5) was employed, except that tryptone was substituted for peptone at a level of 20 mg. per tube. The tryptone was adsorbed at pH 6.6 to 7.0 with Darco G-60 for three 30 minute periods. For *Lactobacillus arabinosus* the medium of Skeggs and Wright (6) was used with the inclusion of adequate calcium pantothenate. In some of the studies with *Lactobacillus arabinosus* PABA was omitted from the medium.

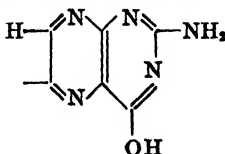
Compounds Used—The synthetic pterins used in these studies have the following structure,² where R and R' are hydrogen, methyl, phenyl, or carboxyl, as well as compounds having phenanthro and acenaphtho groups fused to the pyrimido(4,5-*b*)pyrazine nucleus.



This structure differs from that of the pteridine portion of the folic acid molecule by having an amino group in the 4 position instead of a hydroxyl group. The formulae of these compounds are presented in Table I. 10 per cent glycerol was found to be a somewhat better solvent for these compounds than was water alone. Synthetic folic acid³ was used in all the experiments.

Methods—In studies involving *Streptococcus faecalis* and *Lactobacillus casei* varying amounts of folic acid were added to the medium, as well as increasing amounts of the pterins. In one study with *Lactobacillus arabinosus* increasing levels of the pterins were added to the medium without the addition of folic acid. In a later study with this organism the an-

² The pteridine portion of folic acid has the following structure:

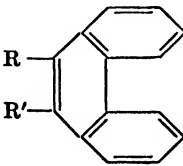
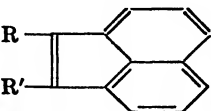


³ The authors are indebted to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for synthetic folic acid.

tagonistic effect of folic acid was demonstrated by showing that folic acid is capable of overcoming the inhibitory effect of the pterin.

In the studies with *Streptococcus faecalis* and *Lactobacillus casei* the assay tubes were inoculated from a liquid culture grown in basal medium containing 100 millimicrograms of Williams' folic acid concentrate.⁴ The

TABLE I
Synthetic Pterins Studied

Compound No.	Structure	Name
1	$R = R' = CH_3$	2,4-Diamino-6,7-dimethylpyrimido(4,5-b)pyrazine
2	$R = H$ $R' = CH_3$	2,4-Diamino-7-methylpyrimido(4,5-b)-pyrazine
3	$R = R' = COOH$	2,4-Diamino-6,7-dicarboxypyrimido(4,5-b)pyrazine
4	$R = H$ $R' = COOH$	2,4-Diamino-7-carboxypyrimido(4,5-b)-pyrazine
5	$R = R' = C_6H_5$	2,4-Diamino-6,7-diphenylpyrimido(4,5-b)pyrazine
6	$R = R' = H$	2,4-Diaminopyrimido(4,5-b)pyrazine
7		2,4-Diaminophenanthro(9,10-e)-pyrimido(4,5-b)pyrazine
8		2,4-Diaminoacenaphtho(1,2-e)pyrimido(4,5-b)pyrazine

inoculum for *Lactobacillus arabinosus* contained the basal medium without added folic acid. *Streptococcus faecalis* and *Lactobacillus arabinosus* were inoculated by loop from 24 hour cultures, whereas *Lactobacillus casei* was centrifuged, washed once with pH 7.0 phosphate buffer, and resuspended in 50 ml. of sterile saline, to which had been added 250 γ

⁴ The 10 per cent folic acid concentrate was kindly supplied by Dr. R. J. Williams, of the University of Texas, Austin, Texas.

of sterile pyridoxal. A sterile 10 ml. serological pipette was used to inoculate *Lactobacillus casei* with a drop of this suspension. *Streptococcus faecalis* and *Lactobacillus arabinosus* were incubated at 30° for 16 to 18

TABLE II
Inhibition of Growth of *Streptococcus faecalis* by Synthetic Pterins

Compound No.	Compound added	Folic acid added	Galvanometer reading*	Anti-bacterial index†	Compound No.	Compound added	Folic acid added	Galvanometer reading	Anti-bacterial index
	γ per 10 ml.	$m\gamma$ ‡ per 10 ml.				γ per 10 ml.	$m\gamma$ per 10 ml.		
1	0	1	41		5	0.002	1	46	
	0	10	14			0.004	1	70	
	0	1000	8			0.008	1	98	8
	2	1	56			2.500	1000	87	
	4	1	70			5.000	1000	94	
	8	1	92	8,000		10.000	1000	100	10
2	20	10	30		6	0.5	1	51	
	40	10	77			1.0	1	85	
	80	10	100	8,000		2.0	1	93	2000
	5	1	51			10.0	10	35	
	10	1	65			20.0	10	75	
	20	1	100	20,000		40.0	10	90	4000
3	50	10	21		7	0.0005	1	60	
	100	10	67			0.001	1	81	
	200	10	87	20,000		0.002	1	96	2
	100	1	51			0.01	10	23	
	200	1	65			0.02	10	50	
	400	1	87	400,000		0.04	10	98	4
4	1000	10	83		8	0.005	1	50	
	2000	10	89			0.01	1	68	
	4000	10	100	400,000		0.02	1	94	20
	25	1	59			0.25	10	26	
	50	1	81			0.50	10	49	
	100	1	100	100,000		1.00	10	96	100
	500	10	46						
	1000	10	73						
	2000	10	91	200,000					

* A reading of 100 represents no growth.

† Ratio of concentration of inhibitor to the concentration of metabolite at which complete inhibition of growth of the organism occurs.

‡ Millimicrograms

hours, and *Lactobacillus casei* at 37° for 20 hours. After the incubation period the growth was measured turbidimetrically with a Coleman spectrophotometer.

Results

Since the inhibitory effect of the various pterins differed with the organism studied, the results with each organism are presented separately.

TABLE III
Inhibition of Growth of Lactobacillus casei by Synthetic Pterins

Compound No.	Compound added	Folic acid added	Galvanometer reading*	Antibacterial index
	γ per 10 ml.	m γ per 10 ml.		
1	0	1	40	
	0	2	28	
	0	5	17	
	50	1	50	
	100	1	65	
	200	1	100	200,000
2	100	5	61	
	200	5	70	
	500	5	90	100,000
	500	1	56	
	1000	1	80	
	1500	1	100	1,500,000
5	1000	5	39	
	2000	5	82	
	4000	5	100	800,000
	20	1	67	
	40	1	80	
	80	1	100	80,000
6	50	5	40	
	100	5	63	
	200	5	100	40,000
	250	1	47	
	500	1	68	
	1000	1	100	1,000,000
	1000	5	29	
	2000	5	51	
	4000	5	100	800,000

* A reading of 100 represents no growth.

Streptococcus faecalis—The results of the studies on *Streptococcus faecalis* are presented in Table II. They show that several of the pterins have an extremely marked antibacterial action for this organism. Competitive antagonism between these pterins and folic acid has been demonstrated over a 10-fold range in concentration for most of the compounds. For the pterin possessing the phenyl group in the 6 and 7 positions, this com-

petitive antagonism has been shown over a 1000-fold range in concentration. The antibacterial index for this compound was found to be the same at the two widely separated concentrations of folic acid.

TABLE IV
Inhibition of Growth of Lactobacillus arabinosus by Synthetic Pterins

Compound No.	Compound added <i>γ per 10 ml.</i>	Galvanometer reading*	
		Plus PABA†	Minus PABA‡
1	0	5	10
	1000	44	79
	2000	79	91
	3000	83	100
	4000	88	100
2	1000	6	22
	2000	11	52
	3000	32	62
	4000	55	74
	5000	71	80
3	1000	5	8
	5000	5	8
4	1000	5	7
	5000	9	30
5	1	5	72
	2	7	83
	5	39	95
	10	74	95
	20	85	96
6	1000	10	28
	2000	25	36
	3000	40	52
	4000	52	67
	5000	60	76

* A reading of 100 represents no growth.

† 1 γ of PABA added per tube.

‡ Medium contains no added PABA.

Studies with 2-amino-4-hydroxy, 2,4-dihydroxy, and 2-mercapto-4-hydroxy analogues of these compounds showed that they had no antibacterial effect on *Streptococcus faecalis*. Some of them, chiefly the carboxy derivatives, exhibited slight folic acid activity. The substitution of a hydroxyl group for an amino group in the 4 position completely nullified the antifolic acid activity of the pterins. Replacing the amino group in the 2 position by a hydroxyl or mercapto group also produced compounds having no inhibitory effect.

Lactobacillus casei—The results of studies conducted with *Lactobacillus casei* are given in Table III. The pterins had much less effect on this organism than on *Streptococcus faecalis*, although both organisms require approximately the same amount of preformed folic acid in the basal medium. The 2,4-diamino-6,7-diphenylpteridine had the greatest inhibitory effect of all the compounds studied. The phenanthro and acenaphtho compounds are so insoluble that it was impossible to obtain

TABLE V

Antagonism of Pterin Inhibition of Growth of Lactobacillus arabinosus by Folic Acid

Compound added*	Folic acid added	Galvanometer reading†	Antibacterial index
γ per 10 ml.	γ per 10 ml.		
0	0	10	
1	0	72	
1	0.05	64	
1	0.5	10	
1	1	8	
2	0	83	
2	0.05	74	
2	0.5	19	
2	1	17	
2	10	13	
4	0.05	100	80
5	0	95	
5	0.5	69	
5	1	71	
5	10	51	
5	50	56	
5	1000	57	
10	0.5	92	20
20	1	96	20

* 2,4-Diamino-6,7-diphenylpyrimido(4,5-b)pyrazine.

† A reading of 100 represents no growth. The medium contained no added PABA.

sufficiently concentrated solutions to show an effect with *Lactobacillus casei*. The carboxy compounds stimulated growth to a slight extent.

Lactobacillus arabinosus—The results of studies with *Lactobacillus arabinosus* are presented in Tables IV and V. Except for the 2,4-diamino-6,7-diphenylpteridine, relatively large amounts of pterins were found to be required to inhibit the growth of this organism. The addition of *p*-aminobenzoic acid caused an antagonism of the pterin inhibition, since 2 to 10 times the amount of compound was needed to prevent the growth of *Lactobacillus arabinosus* when PABA was present. It is evident from the results in Table V that folic acid completely overcame the inhibition

by the pterin at low concentrations of the compound. However, above a certain concentration of the pterin the inhibitory effect was only partially reversible by folic acid. The carboxy derivatives showed little or no inhibition of this organism.

DISCUSSION

The results presented in this report demonstrate that certain synthetic pterins possess strong antibacterial activity which is antagonized competitively by folic acid. In contrast to sulfonamide inhibition which appears to affect only those bacteria which synthesize folic acid, and in opposition to pyrithiamine, thiopanic acid, 3-pyridinesulfonic acid, and many other antimetabolites which are active in inhibiting growth only in those bacterial species which require the metabolite as an essential nutrient, the pterins reported here have the unique capacity of inhibiting the bacteria which synthesize folic acid as well as those requiring the preformed vitamin.

In view of the ineffectiveness of the 4-hydroxy pterins in inhibiting bacterial growth it is apparent that the antibacterial activity of the compounds described in this report hinges upon the presence of an amino group in the 4 position of the pteridine nucleus. Under these conditions the substituent groups present at the 6 and 7 positions have a marked influence upon the antibacterial potency. From the results thus far obtained those pterins possessing large, stable groups at the 6 and 7 positions show the greatest antifolic acid activity.

Woolley (7) has proposed that a compound has the greatest antimetabolite activity when its structural arrangement most nearly coincides with that of the metabolite, except for one essential detail. However, this hypothesis does not hold in explaining the action of the pterins, since the structures such as the phenanthro compound possessing the greater antimetabolite activity are farther removed from the structure of the pterin of folic acid than is that of the 6,7-dimethylpteridine, which showed considerably less antibacterial activity.

A possible explanation of the effects of these compounds is that they act by combining with a specific protein (apoenzyme), thus preventing the normal attachment of folic acid in the formation of an enzyme required for bacterial growth. Since the only functional group common to both the inhibitory pterins and the pterin of folic acid is the amino group in the 2 position, it is possible that union with the apoenzyme occurs at this position. On the other hand, since the antifolic acid pterins also have an amino group in the 4 position, it may be that union in this instance occurs at the 4 position, and is thus responsible for the inhibitory effect. In any case, the effect upon antibacterial activity produced by the groups

on the 6 and 7 positions may be due to their influence upon chemical reactivity of the amino group in the 2 and 4 positions.

The striking dissimilarity in the response of *Lactobacillus casei* and *Streptococcus faecalis* to the pterins suggests that more than one system is involved in the metabolism of folic acid. *Lactobacillus casei* may possess several separate mechanisms, of which one is affected by the pterins, and the others are not. The fact that some pterin inhibition of *Lactobacillus casei* occurs indicates that one of its metabolic pathways is similar to the system functioning in *Streptococcus faecalis*.

SUMMARY

Several synthetic pterins have been shown to possess marked anti-bacterial activity which is antagonized competitively by folic acid. These pterins have the unique capacity of inhibiting bacteria which synthesize folic acid, as well as those requiring the preformed vitamin. A possible explanation for the effects of these compounds is that they act by competing with folic acid for a place in a metabolic reaction essential for the growth of the bacteria.

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A NEW PHOSPHOLIPIDE-SPLITTING ENZYME SPECIFIC FOR THE ESTER LINKAGE BETWEEN THE NITROGENOUS BASE AND THE PHOSPHORIC ACID GROUPING*

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The presence of an enzyme in the raw carrot capable of splitting the phospholipide molecule of the carrot at its ester linkage between the nitrogenous base and the phosphoric acid grouping was postulated in an earlier report from this laboratory (1). The existence of such an enzyme has now been demonstrated. Lecithinases have been found in plant and animal tissues (2-11), but a phospholipide-splitting enzyme specific for the nitrogenous base-phosphoric acid ester linkage has not hitherto been described. This new plant enzyme has been found to act upon plant phospholipides obtained from the carrot and soy bean, but it had no effect on acetylcholine. Although this new plant enzyme liberates choline bound to plant phospholipides, its action is not confined to cleaving this nitrogenous base, for more nitrogen is liberated by the enzyme than can be accounted for by the loss of choline nitrogen.

EXPERIMENTAL

1. *Preparation of Crude Enzyme Extract*—100 gm. of freshly scraped carrots were ground and homogenized in a Waring blender with an equal volume of distilled water, and the mixture allowed to stand at 5° for 12 hours. It was filtered through a Büchner funnel; approximately 150 cc. of extract were then centrifuged. The enzyme extracts used in the experiments described below were never older than 24 hours.

2. *Measurement of Enzymic Activity of Crude Extracts*—An ether solution containing 80 mg. of soy bean phospholipide¹ was transferred to a 50 cc.

* The research which this paper reports was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

¹ A soybean phospholipide was used in all experiments unless otherwise stated. It was prepared from a commercial lecithin by the following procedure: A petroleum ether extract of the phospholipide was carefully washed with water, then concentrated at 55-60° to a small volume (approximately 25 cc.) under reduced pressure (40 to 60 mm.) in an atmosphere of CO₂. This concentrate was taken up in petroleum

glass-stoppered volumetric flask and the solvent removed by evaporation. The evaporated phospholipide was then mixed with 2.5 cc. of enzyme extract and 2.5 cc. of a phosphate buffer at pH 5.9, and the contents of the flask vigorously shaken. 1 drop of chloroform was then added to the emulsified mixture. The mixture was incubated at 25° for 21 hours. At the end of the incubation period, ethyl ether was added to the reaction mixture, and the contents diluted to volume and vigorously shaken. It was necessary to allow about 45 minutes for complete extraction. The ether-soluble fraction was then analyzed for its nitrogen, choline, and phosphorus contents. Nitrogen was measured by the micro-Kjeldahl method, choline by Glick's method (12), and phosphorus by King's method (13). The loss of ether-soluble nitrogen is the index of enzymic activity.

No loss of ether-soluble nitrogen occurred when phospholipides were incubated for 21 hours either in the presence of a phosphate buffer (pH 5.9) or in the presence of the phosphate buffer plus an enzyme extract that had been inactivated by heating it to 95° for 45 minutes.

3. Specificity of Action—The specificity of action of the carrot enzyme is shown in Table I. It is apparent that choline is liberated, but the choline split off the phospholipide molecule does not account for all of the nitrogen lost. Table II shows that neither fatty acids nor glycerophosphoric acid was separated from the phospholipide molecule. The loss of the nitrogenous base is the only degradation suffered by the phospholipide molecule through action of this enzyme. Free fatty acids were measured by the Fairbairn technique (14) and glycerophosphoric acid by Burmaster's method (15).

The enzyme extract possessed no cholinesterase activity, which was tested by the method of Stedman and Stedman (16).

An interesting fact emerging from these studies is the solubility of the enzyme-treated phospholipide in acetone. Upon the addition of acetone to the degraded samples no precipitation occurred, but a turbidity developed which varied proportionately with the loss of nitrogen. It therefore appears that the precipitability of phospholipides by acetone is associated in part with the nitrogenous fraction of the molecule.

4. Kinetic Studies. Order of Reaction—Time-activity studies for this enzyme system, both in buffered and in non-buffered solution, yielded

ether and 3 to 4 volumes of acetone added. The precipitated phospholipides were washed several times with acetone, dissolved in a small volume of petroleum ether, and carefully washed with water to remove non-phospholipide nitrogen. The precipitation procedure was repeated three times and the final precipitate was dissolved in freshly distilled ethyl ether and made to volume. This solution was light red-brown in color and had an N:P molal ratio of 0.90. The commercial lecithin used here was kindly furnished by Mr. M. B. Bieber of the Central Soya Company.

TABLE I
Enzymic Activity of Fresh Carrot Extracts on Plant Phospholipides

Experiment No.	Substrate	Treatment of enzyme extract before incubation	Ether-soluble fraction								Ether-soluble found after incubation, as per cent of original				
			Before incubation				After 21 hrs. incubation								
			P	N	Choline N	N:P, molar ratio	P	N	Choline N	N:P, molar ratio	P	N	Choline N		
			mg.	mg.	mg.		mg.	mg.	mg.						
1	400 mg. carrot phospholipide*	Unheated	11.0	4.79	2.31	0.96	11.0	2.67	0	0.53	100	57	0		
		Heated at 95° 45 min.	11.0	4.79	2.31	0.96	11.0	4.75	2.31	0.96	100	99	100		
2	“ “	Unheated	14.6	7.31	2.70	1.1	14.0	2.36	0	0.37	96	32	0		
		“	14.6	7.31	2.70	1.1	15.2	2.06	0	0.30	103	28	0		
3	520 mg. soy bean phospholipide†	Unheated	13.5	5.77	2.44	0.90	13.5	4.05	1.11	0.66	100	70	46		
		Heated at 95° 45 min.	13.5	5.77	2.44	0.90	13.5	6.45	2.53	1.05	100	110	104		
4	550 mg. soy bean phospholipide†	Unheated	19.2	7.70	2.75	0.90	19.8	4.58	0.53	0.51	103	60	24		
		Heated at 95° 45 min.	19.2	7.70	2.75	0.90	19.2	7.70	2.75	0.90	100	100	100		

* Prepared from steam-treated, dehydrated carrots.

† Prepared from commercial soy bean lecithin.

TABLE II
Free Glycerophosphoric Acid and Free Fatty Acids Formed by Enzymic Action of Carrot Extract on Soy Bean Phospholipide

Experiment No.	Phospholipide added	Total fatty acids in phospholipide added	Total glycerophosphoric acid in phospholipide added	At end of incubation	
				Free fatty acids*	Free glycerophosphoric Acids
	mg.	mg.	mg.	mg.	mg.
1	400	260	64.4	1.4	0
2	400	260	64.4	1.0	0
3	400	260	64.4	1.0	0
4	300	195	44.0	4.0	0
5	300	195	44.0	0.0	0
6	140	91	21.0	9.8	0
7	85	55	12.2	2.5	0

* Calculated as "oleic" acid.

curves representative of a monomolecular reaction. This is shown in Figs. 1 and 2, where $\log (a-x)$ is plotted against t , and where a represents the initial concentration of nitrogen in the intact phospholipide molecule,

x represents the loss of ether-soluble nitrogen from the phospholipide, and t represents time.

The reaction constant k was determined from the equation $k = 1/t(\log(a/a-x))$. The values for k were found to be constant between

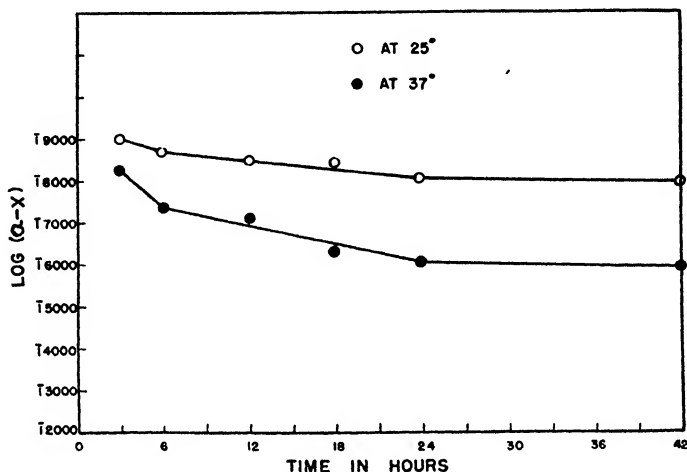


FIG. 1. Time-activity curves for unbuffered phospholipide-enzyme mixtures

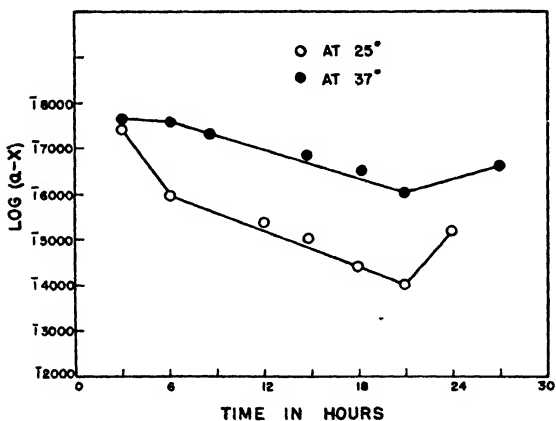


FIG. 2. Time-activity curves for phosphate-buffered phospholipide-enzyme mixtures.

6 and 21 hours. The reaction is a slow one, as is shown by the finding that the cleavage of nitrogen was not complete in 21 hours.

It is of interest that the enzyme system is more active at 25° than at 37° in a phosphate-buffered solution, pH 5.9, while the reverse is true in

unbuffered solutions. The enzyme was less active, however, in the unbuffered solutions. For these reasons all reaction measurements were carried out in the presence of a phosphate buffer at 25°. The use of an acetate buffer yielded results similar to those obtained with the phosphate buffer.

pH-Activity Curve—The procedure outlined in section (2) above was followed. Mixtures of varying proportions of 0.05 M K_2HPO_4 and 0.05 M KH_2PO_4 and varying concentrations of H_3PO_4 were used to prepare solutions with the desired pH. The phospholipide-enzyme buffer emulsions were incubated at 25° for 21 hours. A plot of the activity of this enzyme system at various pH values is shown in Fig. 3. Optimum activity was observed at a pH range between 5.2 and 5.9. Little activity was found below pH 2.0 or above 8.5.

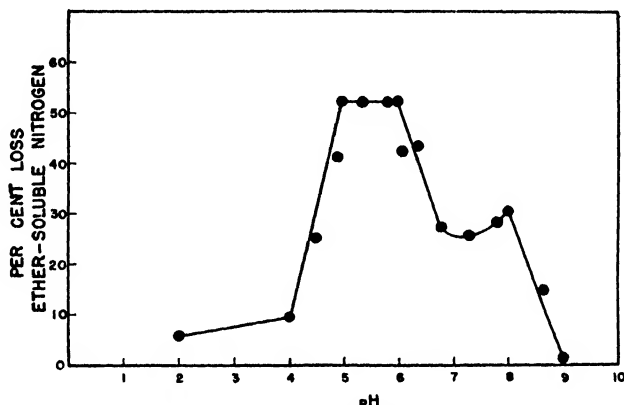


FIG. 3. Enzyme activity at various pH levels

pH-activity curves were also prepared for other incubation periods, namely 10 and 15 hours. For these incubation periods, the amount of activity was, of course, less than at 21 hours, but the plateaux of the curves were still between pH 5.2 and 5.9.

Thermostability—10 cc. aliquots of the crude extract were heated at constant volume for 15 minutes at temperatures ranging from 25–95°. At the end of this time the extracts were immediately cooled to room temperature and the activity measured by the procedure outlined in section (2) above. The results of this experiment are shown in Fig. 4, where activity is plotted against temperature.

This enzyme system is quite thermostable; it retained 10 to 15 per cent of its activity despite being subjected to a temperature of 95° for 15 minutes.

5. Stability of Enzyme System in Steam-Treated Carrots—Two batches of scraped carrots, each amounting to 125 gm., were cut into slices $\frac{1}{8}$ inch

thick. One was steam-treated for 2.5 minutes, the other for 7.5 minutes. The slices were then cooled, homogenized in a Waring blender with an equal volume of distilled water, and stored at 5° for 12 hours. The mixture was filtered through a Büchner funnel and the filtrate tested for activity by the procedure outlined in section (2) above. A similar portion of unheated carrots was used as a control. The results are recorded in Table III.

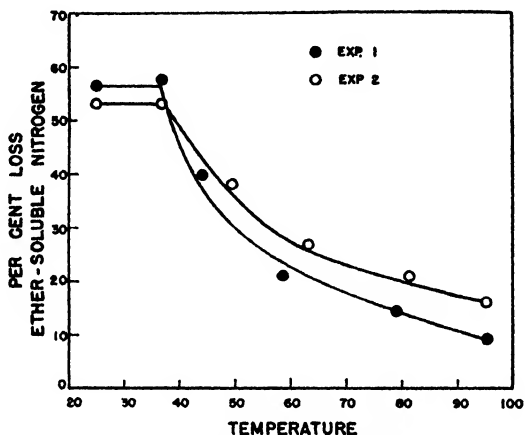


FIG. 4. Thermostability curve

TABLE III

Enzymic Activity of Extract of Steam-Treated Carrots on Soy Bean Phospholipide

Experiment No	Treatment	Per cent phospholipide nitrogen split off
1	Exposed to live steam 2.5 min.	20
2	" " " " 7.5 "	8
3	Unheated	40

Exposure of sliced carrots to live steam for 2.5 minutes reduced the activity of the enzyme system to the extent of about 50 per cent. A heating period of 7.5 minutes produced an 80 per cent reduction in activity.

DISCUSSION

Four types of lecithinases, each acting on a separate ester linkage of the lecithin molecule, were postulated by Contardi and Ercoli in 1932 (17), but the existence of only three of these has hitherto been demonstrated. These are (a) the enzyme found in cobra serum (18-20), which splits off a single unsaturated fatty acid from the lecithin molecule; (b) the enzyme found in rice hulls (17) and *Aspergillus oryzae* (17), which splits off both fatty acids; (c) the enzyme found in *Clostridium welchii* (21), which sepa-

rates the phospholipide molecule at its ester linkage between the glycerol and the phosphoric acid. A plant enzyme capable of attacking only the choline or nitrogen fraction of the phospholipide molecule is described here for the first time.

A phosphorus-containing lipide with a low nitrogen to phosphorus ratio was obtained from raw carrots (1). Since the treatment of the carrot during the isolation of the phospholipide was conducive to enzymic action, it was postulated that an enzyme, probably a lecithinase, was responsible for the degradation of the phospholipide. This interpretation of our earlier findings is confirmed by the data presented here. The action of such an enzyme may well account for the phosphatidic acid-like substances obtained from various plant tissues (22, 23).

SUMMARY

1. A phospholipide-splitting enzyme specific for the ester linkage between the nitrogenous base and the phosphoric acid grouping is shown to be present in the carrot.

2. Maximum activity for this enzyme was observed between pH 5.2 and 5.9 in a 0.05 M phosphate buffer. It showed a high degree of thermostability. It was not completely inactivated when exposed to a temperature of 95° for 15 minutes.

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LIPIDES OF THE FASTING MOUSE

III. PHOSPHOLIPIDE TO NEUTRAL FAT RELATIONSHIP IN THE BLOOD LIPIDES

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The fasting mouse has proved to be a valuable object of study in connection with the complex mechanisms of fat utilization. In the 3 month-old male albino mouse, the utilizable carcass lipides are mobilized and disappear in the course of a 2 day fast (1). On these 1st 2 fasting days, the liver accumulates neutral fat; the total lipide doubles or triples in amount (2). On the succeeding fasting days, the liver total lipide decreases to less than normal amounts. By contrast, the liver phospholipides (in mg. per liver) decrease regularly during the entire fasting period.

The rapid mobilization and burning of the depot fat of the young mouse should be reflected by increases of lipides in the blood stream. In two studies (3, 4), MacLachlan has shown that a moderate lipemia is rapidly established; both the total lipide and the phospholipide levels of the blood increase. The observations of MacLachlan have been extended for a somewhat longer fasting period in the current study. By the use of the radioactive isotope, P^{32} , data on the change in the specific activity of the plasma phospholipides have been observed.

EXPERIMENTAL

The fasting procedure in general was as described before (1). Male albino mice 3 months old, previously maintained on a diet of oats and Purina dog chow, were fasted in individual cages. Water was supplied *ad libitum* and the room temperature was kept at 25°. 24 hours before sacrifice each mouse received intraperitoneally a known amount of P^{32} in aqueous solution as Na_2HPO_4 .

Blood was obtained from the axillary artery according to the method described by Kuhn (5). The whole blood samples were analyzed by standard methods for phospholipide (6) and acetone-soluble lipide, *i.e.* neutral fat and cholesterol (7). Radioactivity determinations were carried out by the usual procedure on the Geiger-Müller scale-of-four counter (8).

Results

Blood Volume—The volume of blood obtained from the fasted mice decreased significantly, relative to the controls, on the 1st day of fasting (Table I). A further significant decrease in blood volume occurred on the 5th day.

Acetone-Soluble Lipide—Although there was a tendency to increase initially (3, 4), the acetone-soluble lipide of the blood showed no marked change from normal until the 5th day, when there was a significant decrease (Fig. 1). This fall in blood lipide may have been related to the depletion of fat reserves.

TABLE I
Data on Blood Lipides and P^{32} Dose per Mg of Blood Phospholipides in Normal and Fasting Mice

	No of mice	Sample		Blood					
				Acetone soluble		Phospholipides			
						Total, 100 ml		Per cent P^{32} dose	
		Mean	S D	Mean	S D	Mean	S D	Mean	S D
		ml	ml	mg	mg	mg	mg		
Normal	15	0.70	0.08	280	60	275	42	0.024	0.006
Fasted 1 day	18	0.59	0.11	289	11	321	90	0.049	0.013
“ 2 days	15	0.56	0.09	298	71	350	63	0.065	0.020
“ 3 “	15	0.55	0.15	282	58	136	103	0.061	0.021
“ 4 “	22	0.58	0.09	279	33	452	105	0.018	0.012
“ 5 “	11	0.45	0.06	236	39	329	52	0.051	0.023

Phospholipide The blood phospholipide content increased markedly and nearly linearly up to the 4th day of fasting, followed by a sharp drop on the 5th day to a value still 20 per cent above the control level (Fig. 2). The increase in blood phospholipide might be explained on the assumption that phospholipide serves as a transport mechanism in the intermediary metabolism of fat. Since complete replacement of the blood phospholipide would require only a small percentage of the total liver phospholipide (9), an increase of the magnitude observed can plausibly be attributed to processes attending fat mobilization.

Percentage P^{32} Dose in Phospholipide The value for the percentage P^{32} dose per mg. of blood phospholipide reached a maximum on the 2nd and 3rd days of fasting. It fell to the control level by the 4th day, but increased again on the 5th day.

It has been shown (9) that labeled inorganic phosphate, when injected intravenously, appears promptly in the liver phospholipides, and within

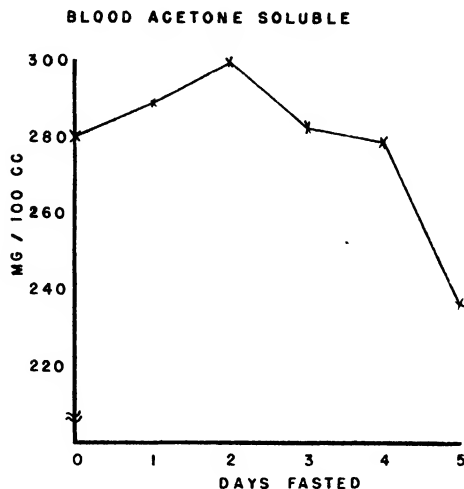


FIG. 1. Acetone-soluble lipid of blood. The initial tendency to increase was followed by a marked fall on the 5th day.

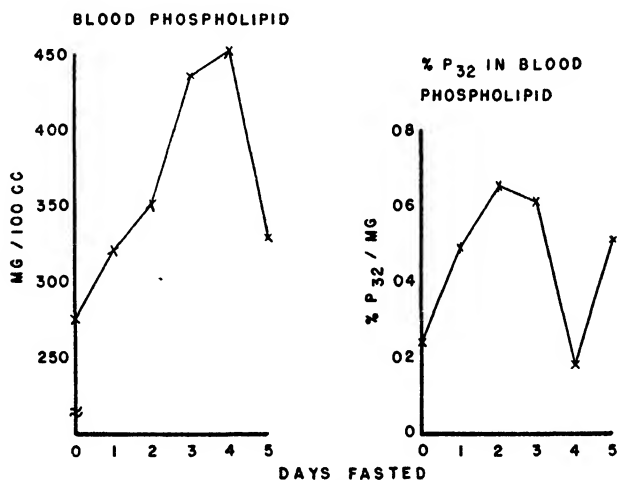


FIG. 2. Blood phospholipide concentration and P^{32} content. The blood phospholipide increased steadily until the 4th day, followed by a sharp decrease on the 5th day. The percentage P^{32} values increased to a maximum on the 2nd day and were elevated again on the 5th day.

4 hours the specific activity of the plasma phospholipide was "similar to that in the liver, which indicates a complete replacement of plasma phospholipide by liver phospholipide." The general agreement between

the values for the per cent P^{32} dose per mg. of blood and liver phospholipides indicates that the same processes may occur in the fasting animal.

DISCUSSION

The data of Table I confirm and extend the observations of MacLachlan that a moderate lipemia occurs in the fasting mouse. Entenman *et al.* (10) found no evidence of such a change in their fasting adult dogs. On the other hand, Bang (11) and Raab (12) have reported a detectable increase in plasma neutral fat in certain dogs. The lipid metabolism in fasting is so rapid in mice that changes may well be found in this species which are not detectable in others. That only a slight rise in total lipid occurred is especially interesting when it is considered that approximately 800 mg. of carcass lipid are mobilized and burned on each of the 1st 2 fasting days. Evidently the mechanism for burning fats is highly efficient.

The increase in phospholipid level, as marked as it is, does not require the addition to the total circulating blood of more than a small fraction of the total phospholipid present in the liver at any one time. For example, the average concentration of blood phospholipid at its peak value on the 4th fasting day was 450 mg. per 100 ml., whereas on the same day the liver contained 3400 mg. of phospholipid per 100 gm. of liver. In other words, the entire circulating blood of the mouse contains perhaps 6 mg. of phospholipid as compared with a content of 20 to 40 mg. of phospholipid in the liver. Consequently, the increase of blood phospholipid is not a large fraction of those phospholipids which might be considered to be readily available.

The percentage P^{32} values fall into a pattern of change with fasting which is similar to that observed in liver phospholipids (unpublished data). It has been found that the percentages P^{32} dose per mg. of liver lecithin and cephalin (determined as in (2)) exhibit a parallel course of change during fasting. The values increase on the first 2 fasting days, are lower on the 3rd and 4th days, and increase again on the 5th fasting day. Numerically, the percentage P^{32} dose per mg. of phospholipid in the blood is more like the values for the cephalin fraction of liver than for the lecithin fraction. (In liver, during fasting the per cent P^{32} dose values for lecithin are uniformly higher than those for cephalin.) In general, it appears that the specific activity of the plasma phospholipid is such as to make the suggestion of origin from the liver a plausible statement.

SUMMARY

A slight lipemia was observed in the early fasting days followed by a decrease on the 5th day. The blood phospholipid concentration increased steadily until the 4th day, then fell on the 5th day. The per cent P^{32}

dose in the blood phospholipides increased to a maximum on the 2nd day and was elevated again on the 5th day.

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PHOSPHATE EXCHANGE AS THE MECHANISM FOR ADSORPTION OF THE RADIOACTIVE ISOTOPE BY THE CALCIFIED TISSUES*

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When phosphate tagged with P^{32} is administered to young rats either by mouth (1, 2) or by intraperitoneal injection (3), some of the P^{32} may be promptly found in the calcified tissues. Ultimately, a considerable proportion of a single dose of P^{32} is present in the skeleton; this amount decreases slowly with time. The promptness has recently been emphasized by the finding of Neuman and Riley (3) that as quickly as 30 minutes after an intraperitoneal administration bone exhibits rapidly increasing percentages of the P^{32} dose. The bone percentages reach maximal values in 12 to 24 hours, at which time 5 per cent (4) or more (2) of the total P^{32} dose may be present in old rats and even greater amounts in young rats (3). It should be emphasized that these phenomena take place much too promptly for calcification or "accretion" of bone to add significant amounts of P^{32} (5). The P^{32} is slowly lost; by the end of the 1st week, a third or more of the peak P^{32} content may have gone from the skeleton; thereafter the rate of loss is slow indeed, so that only an additional one-twelfth may be lost in the 2nd and 3rd weeks combined. Tooth tissues are also found to contain P^{32} , although the rate of deposition is much slower and the amounts deposited are much smaller proportions of the total dose. Thus, from *in vivo* studies, P^{32} appears promptly and in considerable proportions in the bony tissues, from whence it is removed slowly.

In this paper, an attempt is made to describe the P^{32} deposition in bony tissues as an exchange reaction. The idea is not new but its source is not known with certainty (5, 6). For some time, discussion of this phase of bone metabolism has been based on an assumed exchange between bone and blood inorganic phosphate; the data and calculations presented herewith are evidence to support such an assumption.

* The data of this paper are taken in part from the thesis submitted by Marlene Falkenheim to the Graduate School of the University of Rochester, June, 1946, in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry. This work was supported in part by grants from the Carnegie Corporation of New York.

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*Adsorption Studies in Vitro*¹—Surface as a limiting factor in the *in vitro* deposition of P^{32} was proposed (7) following the demonstration that the fixation of P^{32} was described by the Freundlich isotherm (8). Further examination of the adsorption of P^{32} *in vitro* showed that, with longer exposures to the phosphate solutions, P^{32} continued to be adsorbed in a fashion which could be accounted for by assuming diffusion to be a factor (9, 10).

The *in vitro* deposition of P^{32} in powdered bone, dentin, and enamel from aqueous sodium phosphate solutions may be described by the following processes: (a) P^{32} and P^{31} of the solution are unselectively adsorbed by exchange with P^{31} of the surfaces of the minute crystals; (b) "quasi-equilibria" are promptly (2 to 8 hours) reached between the solution and the surface; and (c) diffusion into the powder particles slowly permits more and more crystal surfaces to become available for exchange reactions. This hypothesis will be discussed in some detail.

Procedure

The tissue samples of 50 mg. each of bone, dentin, and enamel (10) of 60 mesh particle size and apatite No 34 (11) were used. Each sample was stirred with 25 ml. of radioactive phosphate solution for increasing lengths of time up to 240 hours. The tests were made at least in quadruplicate and frequently in groups of eight. The P^{32} was obtained as H_2PO_4 and had been prepared by deuteron bombardment of red phosphorus. Radioactivity was determined on scale-of-four Geiger-Müller counters (12). Only three concentrations of Na_2HPO_4 solutions were used; analyzed, they gave values almost precisely 2×10^{-1} , 2×10^{-3} , and 2×10^{-5} M, respectively. Inorganic phosphorus was determined by a modified Fiske and Subbarow procedure. The color was read in a photoelectric colorimeter with a green filter.

Two adsorption procedures were used; in one, the solid-liquid system was agitated by stirring (9), in the other a shaking method was employed (13). After stirring or shaking, the solutions were centrifuged for 10 minutes and decanted. The solids were dissolved in 2 ml. of 3 N HCl, and then diluted suitably with water for radioactivity determinations.

The initial steps of the desorption procedure were exactly the same as those in the adsorption method. After centrifugation, however, the solid samples were resuspended in 25 ml. of an inactive phosphate solution of the same concentration as that used in the adsorption procedure. After desorption for the desired time, the solutions were centrifuged and the solids dissolved in HCl.

¹ The term "adsorption," as used in this paper, neither implies nor excludes a change in the total amount of phosphate at the interface. *Adsorption* is used to describe the transfer of P^{32} from the solution to the solid; *desorption* is the reverse process.

Results

The average percentages of P^{32} adsorbed by bone, apatite, dentin, and enamel from the three Na_2HPO_4 solutions are given in Table I. Various time intervals are indicated for each concentration. It is evident that there are characteristic differences depending (a) on the phosphate concentration, (b) on adsorption time, and (c) on the solid phase exposed to the solutions. In Fig. 1, the typical time courses of the adsorption up to 8 hours for each of the four solids show striking differences in adsorption rates.

TABLE I

Per Cent of P^{32} Adsorbed by Bone, Apatite, Dentin, and Enamel from 10^{-1} , 10^{-3} , and 10^{-5} M Solutions of Na_2HPO_4 in Periods Up to 8 Hours

In each case the average adsorption per cents are given together with standard deviations.

Concentration M	Time hrs.	Bone	Apatite	Dentin	Enamel
2×10^{-1}	1	0.47 ± 0.07	0.91 ± 0.07	0.24 ± 0.07	0.02 ± 0.01
	2	0.56 ± 0.09	0.95 ± 0.08	0.30 ± 0.04	0.03 ± 0.01
	4	0.64 ± 0.07	1.00 ± 0.05	0.40 ± 0.05	0.03 ± 0.02
	8	0.67 ± 0.07	1.01 ± 0.11	0.51 ± 0.05	0.04 ± 0.02
2×10^{-3}	0 25	16 ± 2.2	12 ± 1.8	1.8 ± 0.2	0.4 ± 0.1
	0.5	18 ± 0.8	15 ± 0.9	2.6 ± 0.3	0.5 ± 0.1
	1	23 ± 0.9	18 ± 1.0	3.4 ± 0.7	0.8 ± 0.2
	2	25 ± 1.1	20 ± 1.2	4.5 ± 0.8	1.0 ± 0.0
	4	28 ± 3.8	21 ± 1.4	5.8 ± 0.5	1.2 ± 0.2
	8	31 ± 1.4	26 ± 0.7	6.5 ± 1.2	1.3 ± 0.2
2×10^{-5}	1	72 ± 0.9	39 ± 0.8	16 ± 2.0	2.1 ± 0.6
	2	76 ± 0.8	42 ± 1.1	23 ± 2.3	2.4 ± 0.8
	4	81 ± 0.7	45 ± 1.9	29 ± 2.8	3.8 ± 1.4
	8	83 ± 2.6	46 ± 1.1	38 ± 2.1	5.0 ± 1.0

In Fig. 2, the course of adsorption on bone is illustrated for a period up to 240 hours in contact with a solution of 2×10^{-3} M Na_2HPO_4 , and the curve for desorption is shown covering the same length of time. The samples used for the desorption studies had all previously been through an adsorption exposure of 10 days to a solution of 2×10^{-3} M Na_2HPO_4 .

Exchange Versus Deposition—The *in vitro* deposition of P^{32} in bone has been repeatedly shown under a variety of conditions to give the Freundlich adsorption isotherm. It can easily be shown that this adsorption is not merely a simultaneous deposition of P^{31} and P^{32} in the relative amounts present in the solution. For example, in Fig. 1 the percentages of P^{32} removed from solution by bone rose rapidly, so that in 8 hours about 30 per cent of the P^{32} originally in the 2×10^{-3} M solution was found in the solid. The sample of 25 ml. solution contained 1.55 mg. of P^{31} with suf-

ficient P^{32} to provide about 10,000 counts per minute on our scale-of-four Geiger-Müller counter (12). If 30 per cent of the P^{31} plus P^{32} had been deposited, the solution should have lost 30 per cent of 1.55, or roughly 0.5 mg. of P^{31} . Such a change in concentration would be easily detectable by ordinary chemical means; however, analyses of these solutions gave such values as 1.54, 1.53, 1.56, and 1.52 mg. of P^{31} . Obviously, the amount of P^{31} in the solution had not changed, although a third of the P^{32} was now found on the powdered bone. These facts can be simply accounted for if P^{32} and P^{31} of the solution exchanged with P^{31} of the bone.

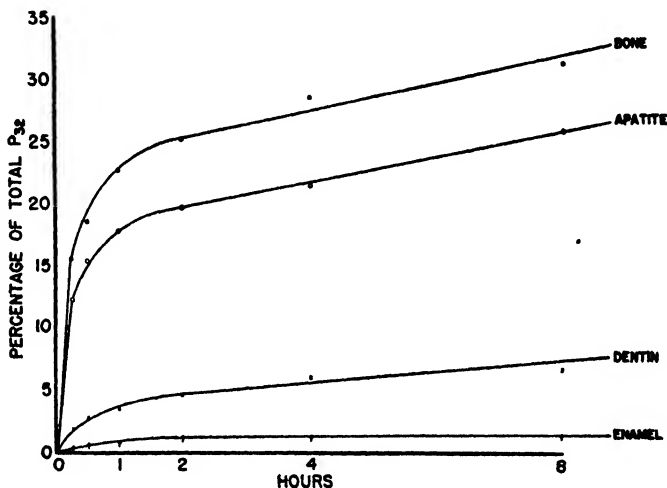


FIG. 1. Adsorption of P^{32} by bone, apatite, dentin, and enamel. Within 2 hours, the rapid phase of adsorption has been succeeded by a slower process. Bone and apatite adsorb much more strongly than dentin and enamel.

Quasi-Equilibria—In Fig. 1 are shown the rates of adsorption of P^{32} from 2×10^{-3} M phosphate solutions by four calcium phosphates. The curves represent at various times up to 8 hours of exposure the percentages of P^{32} adsorbed by the solid phase in contact with solutions which initially contained 100 per cent of the P^{32} .

Each solid phase exhibited a pattern of increasing P^{32} percentages characterized by an initial steep portion of the smooth curve which in an hour or so had risen to about two-thirds of the value obtained after 8 hours of exposure. Later, the more horizontal portions of the curve gave evidence of a much slower, although steady, increase in P^{32} percentage. The rapid achievement of an approximate plateau (a quasi-equilibrium) may be attributed to exchange reactions on those surfaces quickly accessible to the phosphate in solution.

Diffusion—It seems entirely reasonable to assume that the P^{32} adsorption during the first 8 hours is a diffusion process, and a few data may be marshaled to support the assumption. First, many diffusion phenomena are characterized by a proportionality between amounts diffused and the square root of the diffusion times. When the adsorption of P^{32} by bone, dentin, and enamel was plotted against the square roots of times of adsorption, linear relationships appeared (10), indicating that "diffusion is the limiting factor for each tissue" (9). Second, the P^{32} adsorption from 8 hours to 10 days may also involve some undefined diffusion process. Additional support of this suggestion may be found in an experiment on desorption rates. When amounts of P^{32} adsorbed are plotted against the square root of time for this period, the curve approximates linearity. When marked bone samples were prepared by carrying out the adsorption procedure for varying lengths of time, *viz.* 1, 2, 48, and 240 hours, and then when each of these samples was shaken with a P^{31} solution for 8 hours, the percentages of "adsorbed" P^{32} remaining on the bone sample were, respectively, 38, 41, 51, and 65 per cent. Such results would be expected if the longer adsorption exposures had given time for larger proportions of the P^{32} to diffuse into the less accessible portions of the bone particles. In this experiment, the percentages of P^{32} remaining on the bone have a linear relationship to the square root of the *adsorption* times, thereby giving an additional indication that diffusion processes might be involved.

In summary, it may be said that the available data are satisfactorily described by the hypothesis of an exchange reaction. P^{32} exchanges with P^{31} on crystal surfaces as speedily as diffusion will permit.

Reversibility—As has been indicated, the prompt appearance of P^{32} in a P^{31} solution in contact with marked bone is some evidence of reversibility. As expected, the desorption rates and amounts depend on the previous treatment of the marked bone samples, especially on the duration of the adsorption period. In a bone sample that has been exposed for 1 hour to a solution containing P^{32} and then properly washed with water, two major processes occur when the desorption procedure is begun: (1) P^{32} exchanges with the P^{31} solution, and (2) P^{32} in the solution exchanges with P^{31} on the deeper, less available bone crystal surfaces. In a few hours, a plateau of P^{32} content in the solid is attained which represents no critically defined state.

From the studies of adsorption, only small magnitude changes in the P^{32} content of bone occur from day to day from the 6th to the 10th days. Because this period might offer a more nearly fixed condition, adsorption studies were carried out for 10 days, the samples were washed, and desorption exposures were made for an additional 10 day period (Fig. 2). After 10 days the distribution between the solid and the solution should be

approximately the same regardless of whether the P^{32} was in the solid or in the solution initially. The nearly equal distribution shown in the figure arises from the equality of amounts of phosphorus (a) in the solution and (b) in the exchangeable positions in bone crystals.

Exchangeable Fraction--The amount of P^{32} adsorbed by bone and particularly the percentage of the total bone phosphorus which this represents are numbers of physiological interest. A simple calculation gives the fraction of bone phosphorus exchanged; the P^{32} and P^{31} ratio of the solution may be taken as identical with the P^{32} to P^{31} ratio of that solid phosphorus which has taken part in the exchange. The P^{32} content of the solid and the

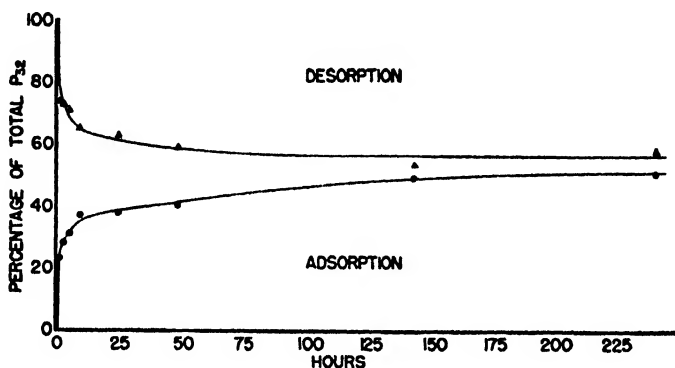


FIG. 2. Adsorption and desorption of P^{32} ; powdered bone. In a 10 day period almost one-half of the P^{32} is adsorbed from a 2×10^{-3} M solution of disodium phosphate onto powdered bone (lower curve). When this bone is replaced in a solution of the same concentration but containing initially no P^{32} , desorption of P^{32} from the solid bone into the solution has removed nearly one-half of the P^{32} in a 10 day period (upper curve). The ordinate represents the percentage of total P^{32} on the bone in each experiment.

solution is determined by counting procedures, the P^{31} content of the solution is obtained by analysis; thus the fraction of P^{31} of bone entering the exchange reaction may be computed. Since the total bone phosphorus is known from direct analysis, the fraction exchanging may be calculated.

The exchanged fraction increases rapidly in the first 8 hours of adsorption; about 5 or 6 per cent has exchanged in an hour and nearly 12 per cent in 8 hours. This rapid increase ceases after 8 hours and is succeeded by a much slower increase; 15 per cent has exchanged in 4 or 5 days and about 19 per cent by the 10th day. Two comments may be added. First, these fractions obtained on powdered bone *in vitro* are closely similar but somewhat larger in each instance than the fractions exchanged in rat bone *in vivo*, as determined by Neuman and Riley (3); they found that about 3 per cent had exchanged in 1 hour and predict that about 15 per cent may ulti-

mately take part in the exchange. It is surprising that phosphorus in blood has nearly as great access to the intact bone in the living animal as a solution of phosphorus has to powdered bone ash in the test-tube. Second, the apparent limit of about 19 per cent in the 10 day adsorption study may have a physical significance. About *one-fifth* of the phosphorus atoms in the bone mineral would be on crystal surfaces if the crystals are hexagonal needles about 10^{-6} cm. long. The possibility of interior phosphorus atoms entering this reaction (at least in the initial phase) is so remote that the fraction of the P^{31} in the surface layer is certainly the physical limit of the exchange.

In phosphate solutions of not too great dilution, the exchangeable fraction may be used to predict the P^{32} distribution when powdered bone is exposed for periods up to 8 hours. Thus, at 2×10^{-1} M, 25 ml. of solution contain 155 mg. of phosphorus. 50 mg. of bone ash contain 0.92 mg. of exchangeable phosphorus. The per cent of phosphorus in the solution phase is 99.3. By coincidence, exactly this figure was found for the percentage of P^{32} in the solution after 8 hour adsorption exposure.

25 ml. of 2×10^{-3} M phosphate contain 1.55 mg. of phosphorus, which is 66.7 per cent of that in the exchange reaction. At 8 hours, 68.9 per cent of the P^{32} was found in the solution. At great dilutions (2×10^{-5}), the rate of exchange is so much slower that the distribution is incomplete after 8 hours, and the results are not directly comparable.

Relative Adsorption by Bone, Apatite, Dentin, and Enamel—Bone and apatite adsorbed relatively large percentages of the P^{32} and the time courses were quite similar, although at each time bone adsorbed somewhat more than apatite. Dentin and enamel adsorbed P^{32} much more slowly, but otherwise in comparable courses; dentin at each time interval adsorbed more than enamel.

In Table I are given the values for the percentages of P^{32} adsorbed by the four solids exposed to three different concentrations of phosphate solutions (*viz.*, 2×10^{-1} , 2×10^{-3} , 2×10^{-5} M) for periods up to 8 hours. The magnitude of the experimental variation is indicated by the standard deviations for each average. Setting the percentage of P^{32} adsorbed by enamel as unity, the relative adsorption by dentin, apatite, and bone may be seen from Table II.

The relative adsorptions maintained a fairly consistent difference regardless of solution concentrations; dentin adsorbed 5 to 10 times as much as enamel, bone adsorbed 16 to 23 times as much as enamel. It appears that the values for apatite may not be comparable, perhaps as a result of increasing hydrolysis with increasing dilution. The apparent tendency toward smaller adsorption percentages for apatite with greater dilution may be an artifact, since the solution after exposure for 8 hours to 50 mg.

of apatite contained 0.6 mg. of phosphorus per 25 ml. instead of the 0.016 mg. of phosphorus present initially. This anomaly is of secondary importance to the major observation; namely, that in these studies, as has been reported before, bone adsorbed more than dentin, which adsorbed more than enamel. That the time curve for adsorption of P^{32} by bone was closely similar to that by apatite in each of the three concentrations studied may be taken as evidence of a fundamental similarity of molecular structure.

Magnitude of Crystal Surface—Powdered bone, dentin, and enamel of the 60 mesh size used are tiny particles of calcium phosphate, but the particle surface is *not* the surface controlling the exchange adsorption of P^{32} . Manly and Levy (8) showed that the location of the adsorption isotherm *ceteris paribus* was independent of particle size; *i.e.*, the same P^{32} adsorption from hot glycol solution was obtained on particles of bone of 40 to 60 mesh size as on particles, all of which passed a 100 mesh screen. In the current study a few samples of powdered bone were exposed to aqueous 2×10^{-3}

TABLE II
Relative Adsorptive Power by Dentin, Apatite, and Bone

Solution concentration	Enamel	Dentin	Apatite	Bone
M				
2×10^{-1}	1	10	25	16
2×10^{-2}	1	5	19	23
2×10^{-3}	1	8	9	16

M phosphate solutions; variation in particle sizes from 60 to 200 mesh produced no alteration of the P^{32} adsorption. Since a decrease in average particle diameter of one-third should result in an increase in particle surface area of the order of nine times, it would appear that some other than the particle surface is taking part in the exchange.

Bony tissues contain crystals of hydroxyapatite laid in a 3 dimensional lattice of protein (*e.g.*, collagen, keratin) fibers. These crystals are sub-microscopic and have been estimated to be of the order of 10^{-6} cm. long (14). Such minute crystals would have a very large surface per gm.

Noel V. Wood, Jr.,² has measured the surface available for adsorption in powdered bone, dentin, and enamel samples by measuring the pressure-volume relationships at very low temperatures and very low pressures of nitrogen gas in contact with the respective powders. On 60 mesh powder samples, he found the following amazing values for specific surfaces: bone 99.3, apatite 51.1, dentin 2.4, and enamel 1.8 sq.m. per gm. It is apparent that an "inner" surface must be active and that the magnitude of the adsorbing surface is extraordinarily large.

² Wood, N. V., Jr., personal communication.

If this surface is to be ascribed to the minute crystals of hydroxyapatite, some rough approximations of the size of crystals to have such total areas may be made. Thus, if the crystals were approximately cubic, the area of N particles would be $N \times 6 \times S^2$ (when S is the length of a side) and the volume would be NS^3 . Since the density of bone is about 2, 1 ml. would have a specific surface of approximately 200 sq.m., or 2×10^6 sq. cm. Simultaneously solving the two equations, $\text{area} = N \times 6 \times S^2 = 2 \times 10^6$ cm., and $\text{volume} = N \times S^3 = 1$ cc., gives an approximate "length" of the hydroxyapatite crystals of 3×10^{-6} cm. This is in excellent agreement with the figure of 10^{-6} cm. obtained by Bale with x-ray diffraction methods. With similar assumptions, the approximate length of an enamel and of a dentin crystal is 10^{-4} cm., which is somewhat larger than the order of magnitude reported by Bale. These calculations

TABLE III
Exchangeable Fractions Determined after 8 Hours Exposure

Solid	Specific surface <i>sq. m. per gm.</i>	Exchangeable fraction	
		Experimental <i>per cent</i>	Calculated <i>per cent</i>
Bone.....	100	12	
Apatite.....	55	7.6	6.6
Dentin.....	2.4	1.8	0.3
Enamel.....	1 8	0.25	0 2

serve only to indicate that the specific surfaces, large as they seem, are of such magnitude as to agree with previously determined dimensions of the hydroxyapatite crystals.

The exchangeable fractions determined after 8 hours of exposure of bone, dentin, and enamel to 2×10^{-3} M phosphate solutions are each of an order of magnitude which corresponds with the specific surface measurements, as determined by the nitrogen adsorption studies (Table III).

In Table III, the specific surfaces and experimentally determined exchangeable fractions are listed. On the basis of the bone values, viz. 100 sq.m. per gm. corresponding to 12 per cent of exchangeable phosphorus, the calculated exchangeable fractions are given as simple proportions. The agreement of estimated and experimental values is striking and seems to strengthen the concept of the surface-limited exchange.

SUMMARY

1. The adsorption of P^{32} by powdered bone, dentin, enamel, and synthetic hydroxyapatite has been measured *in vitro* from several concentrations of aqueous phosphate solution.

2. The process may be described as an exchange in which nearly one-fifth of the phosphorus atoms in the solid bone may ultimately take part.

3. The adsorption presumably occurs on the surfaces of the ultra microscopic crystals of hydroxyapatite; measurements of specific surface area show that large adsorbing surfaces are present.

The authors wish to acknowledge the aid of Dr. Lewis V. McCarty in the discussion of the physicochemical aspects of this work, of Mr. Bernard Sims for maintenance of the Geiger-Müller counters, and of Miss Alice Messinger and Mrs. Irene Nowak for their assistance with the counting. Dr. Robley D. Evans, Massachusetts Institute of Technology, supplied the radioactive phosphorus; Dr. Robert Likins introduced us to Mr. Noel V. Wood, Jr., who performed the nitrogen adsorption measurements.

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STUDIES IN FETAL METABOLISM*

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The origin of the constituents of the mammalian fetus has been the subject of controversy, the literature of which has been exhaustively reviewed by Needham (1, 2) and Huggett (3). Two processes come into consideration: transplacental absorption from the maternal circulation and synthesis in the tissues of the fetus proper.

In regard to the transplacental passage of fatty acids, the most impressive evidence has been the finding of elaidic acid in the fetal fat after the feeding of this abnormal fatty acid to pregnant animals (4, 5). Unequivocal proof for the synthesis of fatty acids (6) and cholesterol (7) in the chick embryo has been adduced, but similar evidence in the mammalian fetus is lacking. There appears to be no reasonable doubt that glucose crosses the placenta, and the relative abundance of fetal glycogen, compared with concentrations in adult tissues, has repeatedly been observed. Whether the latter results from excessive glycogen deposition or lack of glycogen mobilization has not been determined.

The view that epinephrine crosses the placenta has been favored by some workers and contested by others. When epinephrine was administered to pregnant animals, no fall in fetal glycogen content was detected (8). Similarly the transplacental passage of insulin has been the subject of controversy. The most frequent argument has centered about the increase in glucose tolerance incident to pregnancy in the diabetic (9, 10), but this may be ascribed to the large consumption of maternal glucose by the growing fetus and need not indicate passage of fetal insulin into the maternal circulation (11).

The isotopic tracer technique seems to be well adapted to the study of some of these problems. The experiments here reported have been designed to investigate (a) the transplacental migration of certain body constituents, (b) the fetal synthesis of certain constituents, and (c) the con-

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trol exerted on certain fetal metabolic processes by the maternal endocrine state. Pregnant rats, in which the placenta is, as in the human, of the "hemo-chorial" variety (2), were employed in this investigation.

The transplacental passage of fatty acids and of cholesterol has been studied by the administration to pregnant rats of samples of these materials suitably labeled with deuterium, and the isolation of corresponding products from their fetuses.

The synthesis by the fetus of fatty acids, of cholesterol, and of glycogen has been investigated by measurement of the rate at which deuterium appeared in these materials in fetuses which had developed in a medium containing D_2O . This technique has found successful application in the adult animal in the study of the synthesis of fatty acids (12), cholesterol (13), and glycogen (14). In the interpretation of the present results, consideration had to be given to the fact that these materials were being synthesized in the maternal organism as well as in the fetus.

The effects of the injection of epinephrine and of insulin into the maternal circulation upon the quantity of the fetal glycogen have also been studied. If epinephrine crosses the placenta, and the fetal glycogen reserves are susceptible to its mobilizing influence, diminution in the quantity of fetal glycogen should result. Should insulin in the maternal circulation exert a direct action on the fetus, one might anticipate, as in the adult animal, an increase in the fetal glycogen reserves which, if laid down in the presence of D_2O , would be poor in deuterium (15). If, on the other hand, the direct action of maternally injected insulin was confined to the maternal tissues, the effect observed in the fetus would be secondary to the decrease in maternal blood glucose and the increase in the availability of 3-carbon fragments derived from glucose (16). In the light of earlier studies on the adult animal (17), such a shift in the availability of glycogen precursors would result in the deposition of glycogen rich in deuterium when the fetuses were allowed to develop in a medium containing D_2O .

EXPERIMENTAL

Preparation of Isotopically Labeled Materials—The ethyl esters of the fatty acids obtained from linseed oil were reduced in the presence of platinum catalyst in an atmosphere of deuterium (18). The mixed deuterio fatty acid esters melted at 29.5 – 34° and contained 10.0 atom per cent D excess.

Deuteriocholesterol was prepared by the platinum-catalyzed exchange reaction (19) between cholesterol, purified over the dibromide (20), and D_2O . 9.7 ml. of 99.5 per cent D_2O were allowed to react with 12.5 ml. of ordinary acetic anhydride and 600 mg. of platinum oxide (21) were added and reduced with a stream of ordinary hydrogen. After replace-

ment of the gas phase by N_2 , 6 gm. of purified cholesterol were introduced and washed in with 4 ml. of acetic anhydride. The vessel was evacuated, sealed off, and shaken for 3 days at 125–128°.

After removal of volatile solvents, the residue was taken up in 200 ml. of methanol and the platinum removed by filtration. 4.0 gm. of solid KOH were added to the filtrate and the mixture allowed to stand at room temperature for 4 days. On chilling, a crystalline precipitate of cholesterol was deposited, which was filtered off and recrystallized from methanol. After successive drying over $CaCl_2$ and P_2O_5 , the product weighed 2.56 gm. (43 per cent of theory) and melted at 147.5–148°. $[\alpha]_D^{24} = -32.4^\circ$ (2 per cent in $CHCl_3$). D, 3.91 atom per cent excess.

Animals and Diet—Three female albino rats of the Sherman strain, weighing 200 to 300 gm., were placed in a cage with one male rat, and vaginal smears were studied each morning. The presence of spermatozoa was taken as presumptive evidence of pregnancy,¹ and the pregnant rats were thereafter kept in individual cages with wire mesh bottoms.

Except as noted below, they were maintained on a standard diet comprising corn-starch 55 per cent, casein 25 per cent, lard 5 per cent, salt mixture (22) 5 per cent; yeast powder 5 per cent, and roughage (Celluration) 5 per cent. To each kilo of this diet were added 12,000 U. S. P. units of vitamin A, 1700 units of vitamin D, both in percomorph oil, and 100 to 120 mg. of α -tocopherol acetate in sesame oil. These supplements were dissolved in melted lard; the remaining ingredients of the diet were then added in succession, and the whole mixture was homogenized in the ball mill for 24 hours. In all the experiments, water and diet were supplied *ad libitum*, and the animals consumed about 20 gm. of diet daily.

Feeding of Organic Compounds Labeled with Deuterium to Pregnant Rats—On the 18th day of gestation,² one rat was placed on a diet in which the lard was replaced by deuterio fatty acid ethyl esters, weight for weight, and this regimen continued until the 20th day, at which time the animal was killed. To the diet of another pregnant rat, 100 mg. of deuterio-cholesterol were added daily from the 13th to the 20th day of gestation, and the rat then was killed.

Administration of D_2O to Pregnant Rats—On the 18th day of pregnancy, six rats, maintained on the standard diet, were each given a subcutaneous injection of 99.5 per cent D_2O containing 0.9 per cent of NaCl, 1 ml. per 100 gm. of body weight. The drinking water from this point on was replaced by 2 per cent D_2O . This procedure established and maintained a concentration of D_2O in the body fluids of 1.3 to 1.5 per cent. These

¹ Slanetz, C. A., personal communication.

² In describing the age of the fetuses and the duration of the pregnancy, the authors have elected to define zero time as the morning on which pregnancy was diagnosed.

rats were killed at intervals from $\frac{1}{4}$ to 2 days after initiation of the heavy water regimen.

One rat was started on the heavy water regimen indicated above on the day following the diagnosis of pregnancy and was continued on the standard diet with 2 per cent D_2O in lieu of drinking water until the 20th day, at which time the experiment was terminated.

Administration of Epinephrine and Insulin to Pregnant Rats—Two injections of epinephrine, 0.1 ml. of 1:5000 solution per 100 gm. of body weight, were given subcutaneously to two $18\frac{1}{2}$ day-pregnant rats, 3 hours and 1 hour before the animals were killed.

An 18 day-pregnant rat was placed on the heavy water regimen described above and, while receiving the standard diet, was given at 12 hour intervals subcutaneous injections of 5 units of insulin per 100 gm. of body weight. The animal was killed on the 20th day, 1 hour after the last insulin injection.

Isolations and Analyses of Tissue Constituents—At the termination of the experimental periods the animals were asphyxiated with N_2 . The uteri were dissected out and their contents, fetuses, placentae, and membranes were separated from the uterine walls along natural cleavage planes by blunt dissection. The amniotic sacs were preserved intact as far as possible. The number of fetuses encountered per pregnancy varied from 6 to 13, the former occurring in unilateral pregnancies.

The heart, lungs, spleen, and kidneys of the maternal organism were set aside for isolation of a sample of body water (23). The maternal livers, the minced eviscerated carcasses, and the pooled fetuses in each case were digested with hot aqueous 30 per cent KOH, the volume of the reagent approximating the volume of the tissue. After complete disintegration of the tissues had occurred, glycogen was isolated from each portion and purified by previously described methods (14).

Non-saponifiable material was isolated from the aqueous-alcoholic alkaline supernatants from the initial glycogen precipitations by exhaustive extraction with petroleum ether. Cholesterol was isolated as its digitonide, which was dried *in vacuo* over P_2O_5 and paraffin. The weights, as well as the deuterium concentrations, of the cholesterol samples were calculated from data experimentally obtained from the digitonides with the formula $C_{27}H_{46}O \cdot C_{56}H_{92}O_{29}$ as a basis (24).

After separation of the non-saponifiable fractions from the alkaline digests, the solutions were acidified to Congo red paper; fatty acids were extracted with petroleum ether, and dried *in vacuo* over P_2O_5 and paraffin.

The isolations of carcass lipid fractions were carried out on aliquot portions of the alkaline digests.

All deuterium analyses were carried out by the falling drop technique (25).

In the experiments in which epinephrine was injected, as isotope was

not used, isolation of pure glycogen was not necessary and glycogen was determined by the method of Good, Kramer, and Somogyi (26).

DISCUSSION

Passage of Fatty Acids across Placenta—When deuterio fatty acids replaced the normal fatty acids in the diet of a pregnant rat for 2 days, the fatty acids which were isolated from the fetuses contained a significant concentration of deuterium (Table I). A comparison of the deuterium concentrations of the fatty acids fed and those isolated from the fetuses indicates that about 1.5 per cent of the fatty acids recovered from the fetuses were derived, during this period, from the maternal dietary fat. The concentration of D_2O present in the body water was so low that no appreciable introduction of deuterium into fatty acids would have resulted from lipogenesis. From this finding it is concluded that essentially intact fatty acids cross the rat placenta from the maternal to the fetal circulation, though apparently at a slow rate. The present evidence was secured with isotopically labeled fatty acids which, in contrast to claidic acid (4, 5), are indistinguishable from those occurring in nature.

In accord with the results of others (27), the incorporation of deuterio fatty acids into the diet has resulted in their appearance in the liver and depot fat. In the present instance, some 12 per cent of the maternal liver fatty acids and about 1 per cent of the depot fatty acids are estimated to have arisen from the fatty acids of the diet.

Passage of Cholesterol across Placenta—The addition of deuteriocholesterol to the diet of a pregnant rat for 7 days resulted in the appearance of deuteriocholesterol in the fetal sterols (Table I). From the deuterium concentrations of the sterol fed and the sterol isolated from the fetuses, it may be estimated that approximately 10.7 per cent of the cholesterol found in the fetuses was derived from the deuteriocholesterol which had been fed to the mother. As in the preceding experiment, the concentration of D_2O found in the body water was too low to allow for the introduction of appreciable amounts of D into cholesterol by synthetic processes. In accord with the reports of others (28), the addition of deuteriocholesterol to the diet has resulted in its appearance in the maternal liver. After the diet had been enriched with 100 mg. of deuteriocholesterol per day for 7 days, about 40 per cent of the cholesterol recovered from the liver was found to have originated in the diet. A moderate increase in the quantity of cholesterol recovered from the maternal liver appears to have resulted from the dietary supplement.

From the foregoing findings, it is concluded that essentially intact cholesterol molecules cross the rat placenta from the maternal to the fetal circulation.

Experiments in Which D_2O Was Administered to Pregnant Rats—The

study of the synthesis of cholesterol, fatty acids, and glycogen by the rat fetus covered the period from the 18th to the 20th day of pregnancy, a full term in this species being about 21 days. It was found that samples of these materials adequate for isotope analysis could be isolated from the fetuses at this stage of gestation. The durations of the D_2O regimens in the several rats, as well as the gross weights of the tissue fractions, are given in Table II. During the period of observation the average weight per

TABLE I
Placental Permeability to Lipides

Deuterium-labeled compounds were fed to pregnant rats, as indicated, and the rats were killed after 20 days of gestation.

		Deuterio compound			
		Fatty acid esters		Cholesterol	
D concentration, atom %...		10.0		3.91	
Amount fed		5% of diet		100 mg	per day
Duration of feeding, days		2		7	
Total weight of rat, gm....		304		295	
Maternal liver weight, gm.		14.17		11.77	
Weight and No. of fetuses, gm.		69.4 (11)*		49.8 (10)*	
		Tissue content	Deuterium	Tissue content	Deuterium
		per cent	atom per cent	per cent	atom per cent
Fatty acids	Fetal	0.60	0.17	0.81	0.02
	Maternal liver	3.87	1.31	4.61	<0.02
	“ carcass	6.6	0.10	10.7	
Cholesterol	Fetal	0.12	0.02	0.15	0.42
	Maternal liver	0.23	<0.02	0.38	1.58
	“ carcass			0.12	0.34
Body water			0.02		0.01

* The figures in parentheses are the numbers of fetuses per pregnancy.

† Maternal carcass weight is defined as the total body weight minus the weight of pregnancy. All tissue weights are on the wet basis.

fetus increased from approximately 3 to between 4 and 5 gm., and there was no indication that this steady growth was influenced by the number of fetuses per pregnancy. The concentrations of D_2O in the body water samples were all found to lie between 1.30 and 1.46 per cent, and these values were used as a basis for the calculated D concentrations of the isolated tissue constituents, presented in Tables III to VII.

In the last of these experiments, the D_2O regimens was instituted the day following impregnation and was continued up to the 20th day. By this maneuver it could be assured that virtually every molecule that was

synthesized in the fetus throughout its development was synthesized in a medium of heavy water, and it was expected that the isotope concentrations of the fetal tissue constituents, minimally contaminated with

TABLE II
Rats Kept on Heavy Water Regimen

Pregnant rats were injected with D₂O and their body fluids kept enriched with D₂O for the periods indicated, after which they were killed.

Days on D ₂ O	Total weight	Liver weight	No. of fetuses	Fetal weight	Body water D
	gm.	gm.		gm.	atom per cent
18-18½	356	12.98	13	37.3	1.45
18-18½	324	11.64	6	17.6	1.32
18-18½	277	10.57	6	19.7	1.46
18-19	278	12.51	10	34.5	1.40
18-19½	355	12.01	10	43.8	1.37
18-20	312	12.07	10	51.9	1.30
1-20	304	12.69	10	40.9	1.42

TABLE III
Uptake of Deuterium by Fatty Acids in Pregnant Rats Whose Body Fluids Were Enriched with D₂O

Days on D ₂ O	Fetal fatty acids		Liver fatty acids		Carcass fatty acids	
	Tissue content	D	Tissue content	D	Tissue content	D
	per cent	per cent body water*	per cent	per cent body water	per cent	per cent body water
18-18½	0.59	4.4	2.76	7.0	12.8	0.42
18-18½	0.68	6.5	2.50	12.0	10.6	0.76
18-18½	0.60	11.6	2.67	15.2	9.6	0.89
18-19	0.64	14.4	2.32	12.9	9.9	1.7
18-19½	0.74	21.6	3.47	16.9	18.9	0.64
18-20	0.75	21.7	4.49	13.2	11.4	2.8
1-20	0.74	38.9	2.79	20.3	9.2	14.7

* In this and subsequent tables the reported deuterium concentrations have been calculated from the analytical values as

$$\frac{\text{D concentration in constituent}}{\text{D concentration in body water}} \times 100$$

preexisting non-isotopic materials, would approach theoretically maximal values.

Fetal Synthesis of Fatty Acids—Coincident with growth, the per cent of fatty acids in the fetal tissues appears to increase over the period studied (Table III). Starting on the 18th day, when D₂O administration was

begun, a rapid appearance of deuterium occurred in the fetal fatty acids (cf. Fig. 1). Throughout this study less individual variation, in regard both to quantity and isotopic composition, was noted among the constituents of the fetuses than in the corresponding constituents of the adult organism. This we ascribe to the thermal and chemical constancy of the environment in which the fetus exists.

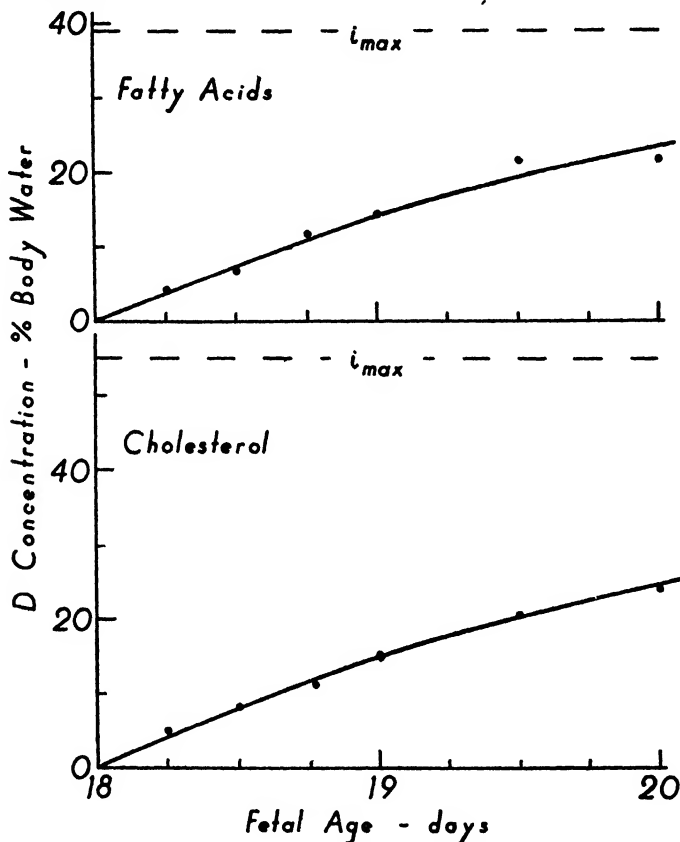


FIG. 1. Incorporation of deuterium into fetal fatty acids and cholesterol, with body fluids enriched with D_2O .

Since the liver is held to be the major site of fatty acid synthesis in the adult mammal, the maternal fatty acid samples richest in deuterium would be expected to be those of the liver. The finding of higher D concentrations in the fatty acids of the fetuses than in those of the maternal livers, in the later samples, is ample indication that lipogenesis proceeds actively in the fetal tissues. It is, of course, true that fatty acids, syn-

thesized in the maternal liver and subsequently transported across the placenta, will contribute to the deuterio fatty acids of the fetus, but this contribution may be expected to be small, in view of our finding of low D concentrations in the fetal fatty acids after the feeding of deuterio fatty acids.

The maximal isotope concentration attained in the fetal fatty acids when the entire development occurred in a medium of heavy water was, experimentally, 38.9 per cent of the body water, in close agreement with other evaluations of the same quantity in the adult animal (13, 14). When D₂O administration was started on the 18th day of pregnancy, the fetal fatty acids reached half of this maximal isotope concentration after about 1½ days (Fig. 1), and it is therefore inferred that at this stage of development approximately one-half of the non-essential fetal fatty acids present had arisen in the preceding 36 hours by synthetic processes. This figure may be compared with the half life of fatty acids previously estimated as 1.9 days in the normal adult rat liver (14), and 9 days in the total rat (29).

The wide scattering of figures pertaining to the incorporation of deuterium into the fatty acids of the maternal organism may be attributed to the disturbing effect of pregnancy upon normal homeostasis. It is noteworthy that this disturbing influence is not transmitted across the placenta to the fetus.

Fetal Synthesis of Cholesterol—Essentially the same arguments apply in the interpretation of the analytical data obtained from the cholesterol samples (Table IV) as have been indicated above in the case of the fatty acid analyses. Since the liver is the only proved site of cholesterol synthesis in the adult organism (30), it is to be expected that at all times the deuterium concentration of the cholesterol of the maternal liver will exceed that of the remaining maternal tissues. The finding in the fetuses of cholesterol richer in deuterium than that of the maternal liver is therefore taken to indicate the fetal synthesis of cholesterol. From the same line of reasoning it follows that the apparently small contribution of maternally synthesized cholesterol which crossed the placenta and was deposited in the fetus could not of itself account for the high concentrations of deuterium observed in the fetal cholesterol samples obtained in the experiments of longer duration.

After the initiation of the heavy water regimen, the D concentration of the fetal cholesterol rose smoothly, approaching a maximal value which was experimentally determined (Table IV) to be about 55 per cent of the D concentration in the body water (Fig. 1). Extrapolation of this experimental curve indicates that the D concentration of the fetal cholesterol would reach half of the maximal value in a period of about 2½ days, from which it may be inferred that, in the rat fetus at this stage of development,

approximately half of the cholesterol found in the fetus had arisen by synthetic processes in the preceding $2\frac{1}{2}$ days. This figure may be compared with the half life of cholesterol in the adult mouse, which has been reported to lie between 15 and 25 days (13), and in the rat liver, which has been reported as 5 to 10 days (30).

Fetal Synthesis of Glycogen—During the 2 days of observation the percentage of glycogen in rat fetuses approximately doubled, representing an increase of about 40 per cent per day (Table V). Expressed as weight of glycogen per fetus, the increase in glycogen content, due to the coincident fetal growth, is even more striking. The D concentration in the samples of fetal glycogen rose smoothly after the initiation of the heavy water regimen, and rapidly approached the experimentally determined maximal value of 27.4 per cent of the body water, achieved in the fetuses

TABLE IV
Uptake of Deuterium by Cholesterol in Pregnant Rats Whose Body Fluids Were Enriched with D₂O

Days on D ₂ O	Fetal cholesterol		Liver cholesterol		Carcass cholesterol	
	Tissue content	D	Tissue content	D	Tissue content	D
	per cent	per cent body water	per cent	per cent body water	per cent	per cent body water
18-18 $\frac{1}{2}$	0.11	5.0	0.18	4.8		
18-18 $\frac{1}{2}$	0.14	8.2	0.20	10.5	0.08	2.5
18-18 $\frac{1}{2}$	0.14	11.3	0.21	11.3		
18-19	0.14	15.4	0.20	13.5	0.11	4.7
18-19 $\frac{1}{2}$	0.15	20.4	0.22	17.7	0.09	3.9
18-20	0.16	24.2	0.27	19.8	0.07	6.7
1-20	0.15	55.4	0.19	45.8	0.11	34.8

of the rat whose body fluids had been enriched with D₂O throughout gestation (Fig. 2). This maximal value, it may be pointed out, is in excellent agreement with extrapolated values previously determined for the glycogen of adult rat liver and carcass (14). On inspection it appears that half of this maximal isotope concentration is attained in a period of from 12 to 18 hours, indicating that half of the glycogen recovered from rat fetuses at this stage of development had been synthesized in this interval. This figure may be compared with earlier evaluations of the half lives of liver and carcass glycogen in the adult rat, 1.0 and 3.6 days respectively (14).

A more satisfactory interpretation of these data is based upon the expected relationship between the rate of increase in the isotope concentration of fetal glycogen, di/dt , and the rate at which glycogen is being synthesized and deposited in the fetus, da/dt (Fig. 3). If 100 gm. of fetus

TABLE V
Uptake of Deuterium by Glycogen in Pregnant Rats Whose Body Fluids Were Enriched with D_2O

Days on D_2O	Fetal glycogen		Liver glycogen		Carcass glycogen	
	Tissue content	D	Tissue content	D	Tissue content	D
	per cent	per cent body water	per cent	per cent body water	per cent	per cent body water
18-18½	0.42	6.0	1.59	1.9	0.13	1.2
18-18½	0.55	12.0	1.38	4.5	0.19	3.9
18-18½	0.54	15.5	2.15	13.4	0.15	3.6
18-19	0.50	18.0	2.36	11.8	0.18	5.1
18-19½	0.72	20.0	0.43	5.3	0.10	3.9
18-20	0.87	22.6	2.09	32.7	0.06	10.4
1-20	0.78	27.4	2.15	27.3	0.14	22.4

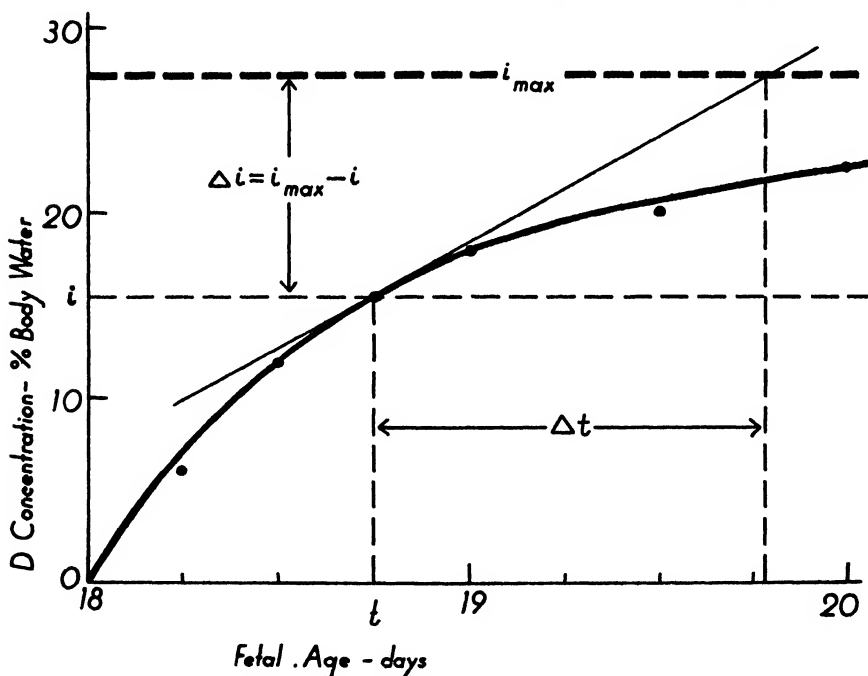


FIG. 2. Uptake of deuterium into fetal glycogen from body water. Construction lines based on the point (t, i) are those referred to in the discussion on the synthesis of fetal glycogen.

contain A gm. of glycogen at isotope concentration i at time t , if da/dt represents the rate at which glycogen is being synthesized and deposited

and $d\alpha/dt$ the rate at which it is mobilized and destroyed, and if, further, it is assumed that each new increment of glycogen has a constant isotope composition represented by i_{\max} , and that the glycogen destroyed has an isotope composition at all times equal to i , then at time $t + dt$

$$(A + dA)(i + di) = Ai + i_{\max}dA - id\alpha$$

from which the relationship given in Fig. 3 may be derived. In view of the fact that A is a variable inadequately defined with respect to time, this expression is not readily susceptible to integration, but a graphic solution is easily obtained. At an arbitrary point (t, i) along the curve (Fig. 2) a tangent has been constructed and perpendiculars have been dropped from the point of tangency as well as from the point of intersection of the tangent with the line $i = i_{\max}$. Since the slope of the tangent, di/dt , is equal to $\Delta i/\Delta t$, and since Δi is, by construction, equal to $i_{\max} - i$, the fraction $(di/dt)/(i_{\max} - i)$ becomes equal to $1/\Delta t$, where

$$\frac{d\alpha/dt}{(i_{\max})} \rightarrow \boxed{\begin{matrix} A \text{ grams} \\ (i) \end{matrix}} \xrightarrow{\frac{d\alpha/dt}{(i)}}$$

$$d\alpha/dt = A \frac{di/dt}{i_{\max} - i}$$

FIG. 3. Relationship between rate of increase of isotope concentration and rate of synthesis of fetal glycogen (cf. the text).

Δt is a measurable line segment. Δt has a value close to 1 day, whence it follows that $da/dt = A/\Delta t =$ about 500 mg. of glycogen synthesized per 100 gm. of fetal weight per day in the $18\frac{3}{4}$ day rat fetus. Stated in another way, the weight of glycogen synthesized and deposited by the fetus per day, at this stage of development, is very nearly equal to the weight of glycogen which it contains. The difference between the rate at which glycogen is synthesized and the rate at which it was found to accrue must be accounted for by the destruction of fetal glycogen, which may roughly be estimated to proceed at about 60 per cent of the synthetic rate.

The rapid turnover of fetal glycogen must be considered in conjunction with the fact that the contents of fat and glycogen in the fetus are of the same order of magnitude, in contrast to the situation in the adult in which the quantity of fat is much larger than that of glycogen. Whereas in the adult animal it was found that the quantity of fatty acids synthesized and deposited each day far exceeded the corresponding quantity of glycogen, the reverse appears to be true in the fetus.

As was noted previously in the discussion of fatty acid synthesis, a wide scattering of points occurred in the deuterium concentrations of the maternal glycogen samples, but whatever the cause of this biological variation in the maternal organism, it was not operative upon the fetuses.

Effect upon Fetal Glycogen of Epinephrine Injected into the Mother—When 18½ day-pregnant rats were killed 1 hour after the second of two subcutaneous injections of epinephrine, the glycogen reserves, both of muscle and liver, were found to have been markedly depleted, as was anticipated (31). In addition, it was found that the fetal content of glycogen had dropped to about 10 to 15 per cent of the normal value (Table VI). This finding is not in accord with the observations of Wertheimer (8), who failed to detect a fall in fetal glycogen after the injection

TABLE VI

Effect of Epinephrine on Fetal Glycogen

The data are taken from rats killed 18½ days after the start of pregnancy. The animals received two injections of epinephrine, 3 hours and 1 hour before being killed. The glycogen contents of the tissues are compared with those of an untreated pregnant rat at the same stage. The figures in parentheses are the numbers of fetuses per pregnancy.

	Total weight	Liver weight	Fetal weight and no.	Glycogen content		
				Fetal	Liver	Carcass
	gm.	gm.	gm.	per cent	per cent	per cent
Untreated control	324	11.64	17.6 (6)	0.55	1.38	0.19
Epinephrine injected	298	10.31	20.2 (8)	0.045	0.017	0.002
	345	13.61	29.0 (10)	0.088	0.082	0.002

of epinephrine into pregnant rats. The apparent discrepancy may be due to the lapse of several hours which intervened between the injection and the termination of his experiment.

The most plausible explanation for the observed fall in fetal glycogen is that a portion of the epinephrine which had been injected into the maternal tissues crossed the placenta and, in the fetuses, stimulated the mobilization of glycogen, as it is known to do in adult tissues. Regardless of the question of the capacity of the fetal adrenal medulla to liberate epinephrine, it may be inferred that the fetal glycogen stores are to some extent subject to the influence of epinephrine present in the maternal circulation.

Effect upon Fetal Metabolic Processes of Insulin Injected into the Mother—The repeated injection of insulin into a pregnant rat on the D₂O regimen resulted in a marked decrease in the quantity of maternal liver glycogen, in accord with reports of others (32). Wherea striking changes in the

composition of the fetuses and in the D concentrations of the fetal constituents were not found, comparison with the data obtained from an untreated pregnant control rat revealed a slight decrease in the quantity of fetal glycogen and an increase in the concentrations of D in fetal glycogen, fatty acids, and cholesterol (Table VII). These changes cannot be attributed to any known *direct* action of insulin, but may well have resulted secondarily from an increased availability in the maternal blood of 3-carbon derivatives of glucose (16) and decreased availability of glucose itself. Both fatty acids and cholesterol are currently pictured as being

TABLE VII
Effect of Insulin on Fetal Metabolism

The data are from rats killed 20 days after the start of pregnancy. The body fluids were kept enriched with D₂O for 2 days prior to sacrifice. A pregnant rat received injections of insulin every 12 hours over a period of 2 days, and the data are compared with those of an untreated pregnant rat at the same stage. The figures in parentheses are the numbers of fetuses per pregnancy.

		Untreated		Insulin-treated	
Total weight, gm.		312		365	
Liver weight, gm.		12.07		12.22	
Fetal weight and no. , gm.		51.9 (10)		50.7 (10)	
Body water, atom % D		1.30		1.32	
		Tissue content	D	Tissue content	D
		per cent	per cent body water	per cent	per cent body water
Glycogen	Fetal	0.87	22.6	0.63	29.2
	Liver	2.09	32.7	<0.12	*
	Carcass	0.06	10.4	0.10	8.6
Fatty acids	Fetal	0.75	21.7	0.76	27.0
	Liver	4.49	13.2	3.96	12.7
	Carcass	11.4	2.8	11.3	3.1
Cholesterol	Fetal	0.16	24.2	0.14	33.2
	Liver	0.27	19.8	0.20	25.0
	Carcass	0.07	6.7	0.09	8.2

* Sample too small for analysis.

capable of arising from glucose via such small fragments (33, 34), and in this type of isotope experiment glycogen has been found to be richer in deuterium when small fragments such as lactate, rather than glucose, are the chief precursors (17). From the present experiment no evidence could be derived to support the view that maternal insulin exerts any direct action upon fetal metabolic processes.

SUMMARY

The feeding of deuterio fatty acids and deuteriocholesterol to pregnant rats in the latter part of gestation resulted in the appearance of the cor-

responding deuterio compounds in the fetuses, proving that these compounds cross the rat placenta.

Enrichment of the body fluids of pregnant rats with D_2O resulted in the rapid incorporation of D into fetal glycogen, fatty acids, and cholesterol at rates indicating that these compounds are synthesized in the fetal organism.

The data indicate that, in the rat fetus approaching the 20 day stage of development, half of the fetal glycogen had arisen by synthesis in 12 to 18 hours, half of the fetal fatty acids in about $1\frac{1}{2}$ days, and half of the fetal cholesterol in about $2\frac{1}{2}$ days. The quantity of glycogen synthesized and deposited in the fetus per day was found to be approximately equal to the total amount of glycogen present at this stage of development; i.e., some 500 mg. per 100 gm. of tissue. The synthetic rates in the fetus are all appreciably higher than the corresponding rates in adult rats.

Injection of epinephrine into the pregnant rat caused a drop in the content of fetal glycogen, suggesting that epinephrine crosses the placenta and that fetal glycogen is susceptible to its glycogenolytic stimulus.

The injection of insulin into a pregnant rat containing D_2O in its body fluids caused a slight increase in the deuterium incorporated into fetal glycogen, fatty acids, and cholesterol. This is attributed not to any direct action of insulin in the fetus, but rather to decreased availability of maternal glucose and increased availability of small fragments derived therefrom.

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MICROBIOLOGICAL DETERMINATION OF THREONINE IN PROTEINS AND FOODS

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In only eight of the sixteen articles describing media for the determination of threonine (1-16) have the authors actually developed standard curves and assayed materials. Five of these eight publications outline the use of *Streptococcus faecalis* (5, 7, 10, 12, 15), two explain the use of *Lactobacillus arabinosus* 17-5 (8, 9), and one describes the use of *Lactobacillus fermenti* 36 (13).

In the laboratory, attempts were made to develop standard curves with *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* P-60 with media previously used for methionine and lysine (17, 18). A satisfactory blank could not be obtained with *Lactobacillus arabinosus*. With *Leuconostoc mesenteroides* a sigmoidal curve was obtained. The addition of β -alanine, inositol, ammonium chloride, asparagine, pimelic acid, and xanthine did not change the nature of this curve. However, the addition to or withdrawal in part of the serine from the medium caused an increase or decrease of the sigmoidal part of the curve, but at no concentration of serine was the inflection eliminated entirely.

A satisfactory standard curve was prepared by using *Streptococcus faecalis* and the medium previously used for methionine (17), with the exception that pyridoxine was replaced by 400 γ of pyridoxamine per liter (19).

EXPERIMENTAL

*Streptococcus faecalis*¹ was used in the assays described in this paper.

Basal Medium—The basal medium was the same as that described in a previous paper (17), with the exception noted above.

Assay Procedure—The procedures followed for the cultures, inoculum, and preparation of samples were identical with those described for the assay of lysine (18).

Preparation of Threonine Standards—As Stokes and associates (5) have shown that only one-half of the racemate is active, threonine solutions ranging in concentrations from 5 to 100 γ per ml. of the optically active

¹ Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C., marked *Streptococcus faecalis* No. 9790.

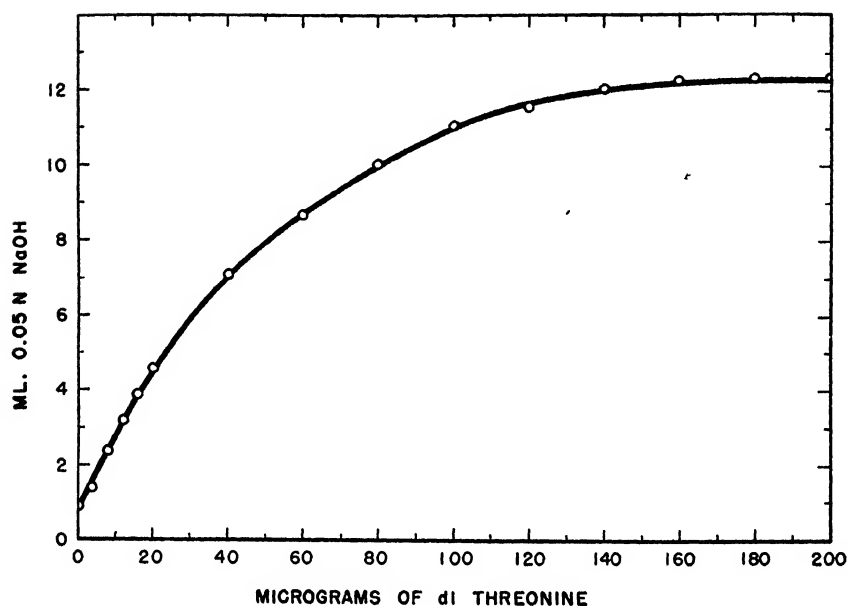


FIG. 1. Standard titration curve

TABLE I

Recovery of Threonine Added to Protein Hydrolysate

Protein hydrolysate	Threonine				
	In hydrolysate*	Added	Total	Found	Recovery
	γ	γ	γ	γ	per cent
Casein	4.5	10	14.5	15.0	103
	9.0	10	19.0	19.0	100
	13.5	10	23.5	23.5	100
	18.0	10	28.0	28.0	100
Corn, whole yellow	2.35	20	22.35	22.0	98
	4.70	20	24.70	24.30	98
	7.05	20	27.05	27.50	102
Ox muscle	5.3	10	15.3	15.5	101
		20	25.3	25.5	101
		30	35.3	35.7	101
		40	45.3	45.8	101
Soy bean flour	4.5	20	24.5	24.8	101
	9.0	20	29.0	29.5	102
	13.5	20	33.5	34.0	102

* Uncorrected for moisture and ash.

isomer were prepared by weighing the DL compound. The standard curve prepared with these solutions is shown in Fig. 1. Titration values were

TABLE II

Threonine Content of Some Proteins and Foods Determined at Different Assay Levels

Material	Protein assay level	Threonine found*	Material	Protein assay level	Threonine found*
	γ	<i>per cent</i>		γ	<i>per cent</i>
Corn, whole yellow	500	0.46	Glycinin	200	2.75
	1,000	0.47		300	2.76
	1,500	0.47		400	2.80
	2,000	0.49		500	2.77
	2,500	0.46		800	2.75
	5,000	0.45		1,200	2.71
	7,500	0.45		1,600	2.81
	10,000	0.48			(2.76)
Cottonseed flour		(0.47)	Rye, whole	1,000	0.44
	200	2.10		2,000	0.44
	400	2.12		2,500	0.44
	600	2.10		3,000	0.43
	800	2.12		4,000	0.44
Edestin		(2.11)	Wheat, whole	5,000	0.43
	100	4.0		7,500	0.43
	200	4.1		10,000	0.45
	300	4.1			(0.44)
	400	4.0		500	0.50
		(4.05)		1,000	0.50
				1,500	0.50
				2,000	0.51
				2,500	0.52
				5,000	0.51
				7,500	0.53
					(0.51)

* Uncorrected for moisture and ash. The figures in parentheses represent averages.

TABLE III

*Reproducibility of Threonine Content by Separate Assays**

Material	Assay 1	Assay 2	Average
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein.	4.52	4.51	4.51
Corn, whole yellow.	0.47	0.47	0.47
Ox muscle	5.32	5.31	5.31
Rye, whole	0.44	0.44	0.44
Soy bean flour.	2.25	2.26	2.25

* Uncorrected for moisture and ash.

not altered by the addition to the medium of 1.2 mg. of any of the nineteen amino acids.

Recovery of threonine added in different proportions to hydrolysates of casein, ox muscle, soy bean flour, and whole yellow corn gave results well within the experimental error for this type of assay (Table I).

TABLE IV
Threonine Content of Some Proteins and Foods *

Percentages calculated for ash- and moisture-free material.

Material	N	Threonine	Values from literature
	<i>per cent</i>	<i>per cent</i>	
Arachin . . .	18.30	2.89	2.65 (21)
Casein. . .	16.07	4.80	4.2 (5), 4.65 (10), 4.42 (13), 4.00 (22), 4.6 (23)
Coconut globulin	17.42	4.06	
Conarachin . .	18.20	1.93	2.02 (21)
Cottonseed globulin	18.00	3.96	2.9 (24)
Edestin . . .	18.55	4.34	
Gelatin (Bacto)	18.32	2.10	2.0 (5), 1.9 (8), 1.9 (10), 1.4 (25), 2.0 (26)
Glycinin . . .	17.30	3.00	
Lactalbumin	15.39	5.37	5.0 (7), 5.6 (8), 5.24 (22), 5.20 (27)
Ovalbumin (crystalline)	15.98	4.48	3.6 (5), 4.3 (27), 4.6 (28)
Ox muscle . . .	16.00	5.80	5.4 (8), 5.7 (27), 4.6 (28)
Peanut, total globulins	18.01	3.24	
Phaseolin (navy bean).	16.07	4.16	
Wheat bran globulin. .	17.76	3.50	
Zein . . .	16.00	2.62	2.3 (26), 2.5 (29)
Barley, pearled	1.86	0.42	0.26 (10)
Brazil nut meal	9.03	1.60	
Corn germ, defatted	3.93	1.09	1.16 (27)
“ whole yellow . . .	2.22	0.54	0.64 (7), 0.44 (10), 0.47 (27)
Cottonseed flour	10.36	2.38	1.78 (10), 1.95 (27)
Egg, whole, dried	8.11	3.00	2.51 (27)
Milk, dry, skim	6.57	2.15	1.64 (5), 1.97 (12), 2.01 (23), 1.89 (27)
Oatmeal . . .	2.73	0.62	0.61 (27)
Peanut flour. . .	10.15	1.93	0.95 (27)
Peas, black-eyed	4.15	1.09	
Rice, white . . .	1.26	0.28	0.31 (27)
Rye, whole	1.98	0.49	0.38 (5), 0.27 (10)
Soy bean flour . .	8.85	2.53	2.16 (5, 7), 2.57 (10), 2.20 (27)
Wheat, germ, defatted.	6.50	1.63	1.55 (27)
“ whole	3.07	0.57	0.49 (5), 0.48 (10), 0.63 (27)
Yeast, dried, brewers' .	7.71	2.54	2.46 (5), 2.41 (27)

Table II shows values found for whole wheat, whole yellow corn, whole rye, glycinin, cottonseed flour, and edestin determined at different assay levels. Data on reproducibility of the amount of threonine found in soy bean flour, casein, whole yellow corn, ox muscle, and whole rye are given in Table III.

The threonine values² (Table IV) found for the thirty-one proteins and foods assayed agree well with those reported by others using both microbiological and chemical methods.

SUMMARY

A microbiological method is described for the determination of threonine with *Streptococcus faecalis*. The procedure has been applied to the assay of thirty-one proteins and foods with results which agree well with those obtained by both microbiological and chemical methods.

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² The sources and preparation of the samples assayed are given in a previous publication (20).

SEDIMENTATION, VISCOSITY, AND ELECTROPHORETIC STUDIES ON PURIFIED LEE INFLUENZA VIRUS PREPARATIONS*

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Crude preparations of PR8 influenza virus, obtained by high speed centrifugation of the chorioallantoic fluid of infected chick embryos, contain an impurity which is similar to substances elaborated normally by uninfected embryos. Electron microscope and ultracentrifuge measurements have shown that the impurity is smaller in size than the virus (1-7), while viscosity determinations revealed that it is much more viscous (3, 4). Electrophoretic tests indicate that it is present to the extent of 10 to 20 per cent (8). Removal can be effected by selective adsorption of the virus on chicken red blood cells and subsequent elution therefrom (1, 9), by special methods of high speed centrifugal fractionation (4), or by electrophoretic fractionation (8). Purified virus thus obtained still contains, however, as an integral part of its structure, at least 20 per cent of antigenic groupings characteristic of the impurity (9).

Particles of a size intermediate between those of the normal material and those of the virus occur in centrifugally isolated preparations of Lee influenza virus (2, 10) and also, in some instances, of PR8 virus (2, 11). Evidence has been presented which suggests that such particles may represent degradation products (2, 10) or, possibly, precursors (11) of the virus.

Further studies of Lee virus with the aid of sedimentation, viscosity, and electrophoretic measurements are presented in this paper. Purification of crude preparations was attempted by centrifugal and electrophoretic fractionation procedures and by the adsorption-elution method, and the physical chemical and biological properties of the different fractions were investigated.

Materials and Methods

The Lee strain of influenza virus was supplied by Dr. T. Francis, Jr. Fertile eggs that had been incubated for 10 days at 39° were each inoculated

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research. It was carried out under the supervision of Dr. W. M. Stanley to whom acknowledgment is due for much valuable discussion and criticism. The present address of the author is The Institute for Cancer Research, Philadelphia.

with 0.1 ml. of infectious chorioallantoic fluid at a dilution of 10^{-4} to 10^{-5} , incubated for a further 48 hours at 36° , and chilled overnight at 4° . The shells above the air sacs were removed with the aid of a small circular saw, the chorioallantoic membranes were torn, and the fluids were poured through glass wool in a funnel. Good yields of fluid free from suspended material were obtained.

Agglutination titrations of chicken red blood cells were carried out by the method of Hirst and Pickels (12), as modified in this laboratory (13). Infectivity titers were determined with the use of chick embryos (14, 15).

Adsorption of Lee virus on chicken red blood cells and elution therefrom, originally described by Hirst (16), was carried out on a large scale by the procedure described by Sharp and coworkers (10), except that a 1.5 per cent suspension of red cells was used for the adsorption and 0.1 M phosphate buffer at pH 7.1 for the elution. Also, except where indicated otherwise, a single adsorption and elution were performed. When centrifugally isolated virus was subjected to adsorption, it was first diluted to 0.05 mg. of material per ml. in 0.1 M phosphate buffer, for higher concentrations resulted in losses. After elution, the virus was subjected to two high speed centrifugation cycles as described below to remove low molecular weight materials arising from the blood cells (17). Virus purified by the red blood cell procedure was designated "RBC" virus.

Sedimentation of the virus from infectious chorioallantoic fluid was carried out by two 15 minute cycles at 24,000 R.P.M. (5, 17). In some cases, the Sharples centrifuge was used for the first concentration step (17), followed by a single sedimentation in the quantity ultracentrifuge. The final pellets were dissolved in 0.1 M potassium phosphate buffer at pH 7.1, and the solution was freed from aggregated material by centrifugation for half an hour at 3000 R.P.M. in a Swedish type of angle centrifuge. Nitrogen was determined by a micro-Kjeldahl method (18) and converted to values for virus on the basis of 9.7 per cent nitrogen in the virus (19). For further purification with the aid of high speed centrifugation, certain preparations of the "crude" virus were carried through the special fractionation procedure described for Preparation 7 of Lauffer and Stanley (4). The fractions obtained were designated the "heavy" and "light" fractions.

Analytical ultracentrifuge measurements were carried out in a Bauer and Pickels centrifuge (20) equipped with a Svensson optical system (21). Observed sedimentation rates were corrected to standard conditions of water at 20° as the theoretical medium. The value, 0.863 ml. per gm., for the partial specific volume of anhydrous Lee virus preparations (22) was used in the correction. Tracings of sedimentation diagrams were made for comparative purposes and for calculations of standard deviations of sedimentation rates within individual virus preparations. These were drawn midway between the top and bottom edges of the Svensson curves.

Viscosity measurements were made in 0.1 M potassium phosphate buffer at pH 7.1 with a 1 ml. Ostwald viscometer, as described by Lauffer and Stanley (4).

Electrophoretic methods were applied essentially as described previously in studies of PR8 virus (8). Pellets of Lee virus preparations to be used for micro electrophoresis experiments were suspended in 0.02 M potassium phosphate buffer at pH 7.1 instead of 0.02 M veronal buffer at pH 7.4, since, unlike the PR8 strain, the Lee strain was not readily dispersed in the latter medium. Further, because of the anomalous behavior of the Lee virus preparations in the veronal buffer, moving boundary electrophoretic studies were carried out only in 0.1 M potassium phosphate buffer at pH 7.1. The field strength in the moving boundary studies was maintained at 1.35 volts per cm. or lower.

Results

Yield and Chicken Red Blood Cell Agglutinating Activity of Starting Material—Infectious Lee chorioallantoic fluids tested in four different experiments possessed chicken red blood cell agglutination titers of 54, 82, 82, and 95 units per ml. The average value, 78 units per ml., was roughly one-fourth to one-half that of PR8 chorioallantoic fluid (18). Yields of five preparations of crude virus obtained by high speed centrifugation were 0.029, 0.031, 0.034, 0.043, and 0.050 mg. per ml. of chorioallantoic fluid, averaging 0.037 mg. per ml. The average chicken red blood cell agglutinating activity of five crude virus preparations was 1860 units per mg., based on individual values of 1460, 1640, 1900, 2080, and 2210 units per mg. The yields of the crude Lee virus were approximately two-thirds and the chicken red blood cell agglutination titers approximately one-half those obtained under optimum conditions for PR8 virus (18).

Sedimentation Studies—Sedimentation diagrams of different fractions of Lee virus preparations are shown in Fig. 1. The crude preparation employed as starting material reveals two principal components and appears similar to that of Friedewald and Pickels (2). The light fraction contains an increased proportion of the more slowly sedimenting component, while the corresponding heavy fraction is practically free from it. The RBC preparation also appears free from the slowly sedimenting component. The sedimentation constants of the rapidly sedimenting components of the crude, light, heavy, and RBC preparations were 703, 648, 723, and 746 Svedberg units, respectively, the variation in which will be referred to later.

The relationship between concentration of virus and reciprocal of the sedimentation rate is shown graphically in Fig. 2, in which a straight line was fitted to the experimental data by the method of least squares. RBC virus was used for these measurements since, from the above studies together with others yet to be presented, it appeared the purest of the various frac-

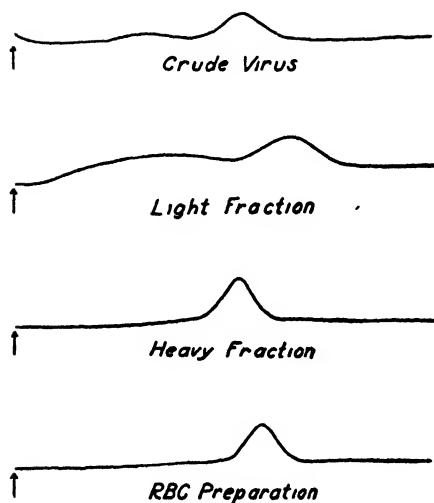


FIG. 1. Tracings of Svensson sedimentation diagrams of Lee influenza virus fractions. Positions of boundaries were chosen at which the relative homogeneities of the different fractions were best illustrated, and are not indicative of differences in sedimentation rates. Concentrations of virus were approximately 4 mg per ml. The arrows represent the positions of the meniscus.

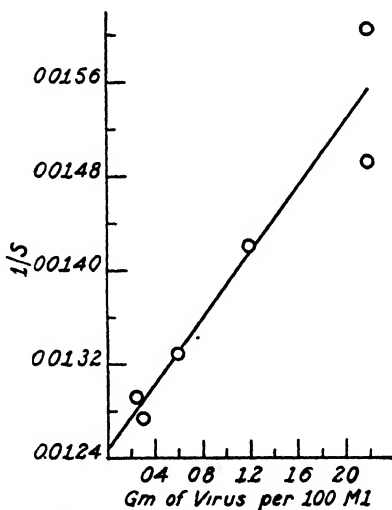


FIG. 2. Relationship between concentration and the reciprocal of the sedimentation rate of a preparation of Lee influenza virus.

tions tested. The sedimentation constant at infinite dilution, determined from the graph, was 802 Svedberg units.

Particles which sediment at a rate as high as 800 Svedberg units would be expected to diffuse extremely slowly. The spread in the boundaries during sedimentation is, therefore, indicative of the existence of a family of particles of slightly variable size or density. The standard deviations of the distributions of the sedimentation rates for the families of particles represented by the heavy fraction and the RBC preparation were calculated to be 7.0 and 6.7 per cent, respectively, of the mean rate. In terms of particle diameters, these figures corresponded to standard deviations of 3.4 and 3.3 per cent. The degree of homogeneity of the purified Lee virus preparations is thus slightly higher than that of centrifugally purified PR8 virus preparations (4).

The chicken red blood cell agglutination titers of the crude, light, heavy, and RBC fractions shown in Fig. 1 were 2080, 2010, 2620, and 3010 units per mg., respectively. The data for the titers show, first of all, the efficacy of centrifugal fractionation in increasing the agglutinating activity of crude Lee virus preparations. This was confirmed in another experiment in which the activities of the crude, light, and heavy fractions were 1900, 1280, and 2600 units per mg., respectively. The data also indicate that the RBC virus possessed a titer even higher than that of the heavy fractions. This finding was checked by a further experiment in which an RBC virus preparation containing 2900 units per mg. was obtained directly from a heavy fraction containing only 2520 units per mg. In other experiments, RBC virus of 3020 units per mg. was obtained from crude virus of 2210 units per mg., and RBC virus of 2770 units per mg. was prepared directly from infectious chorioallantoic fluid. The agglutination tests as a whole indicate that the slowly sedimenting material possesses a lower biological activity than the rapidly sedimenting component. Furthermore, the higher activity of the RBC preparations, compared to those of the heavy fractions, suggests the presence of heavy, inactive material in the latter. This can be explained by the fact that an impurity of about the same particle weight as the virus would not be removed by the centrifugal fractionation but would be removed by the more specific adsorption-elution method.

If it is assumed that the chicken red blood cell agglutination titer of the RBC preparations represents that of pure virus and, further, that the presence of impurity does not affect the titers, it can be estimated from the activities of the different preparations that the crude virus contained somewhat over 30 per cent impurity, of which two-thirds was of small particle size and one-third was of a size comparable to that of the virus.

Viscosity Studies—The intrinsic viscosities of the crude, light, heavy, and RBC preparations shown in Fig. 1 were found to be 36.4, 46.4, 33.4, and 8.64, respectively. It may be seen that preparations with lowest sedimentation rates possess the highest viscosities, and vice versa. The results

suggest that the slowly sedimenting component contributes more to the viscosity than the rapidly sedimenting one. Furthermore, since the RBC preparation possessed by far the lowest viscosity, it is probable that the more viscous material represents impurities, as was the case with the PR8 virus (4). In another experiment, a crude preparation of Lee virus gave an intrinsic viscosity of 42.1, the RBC preparation only 9.86. To determine whether the viscosity of this RBC preparation might be decreased further, it was subjected to a second adsorption and elution with red blood cells, followed by high speed centrifugation. The final product showed,

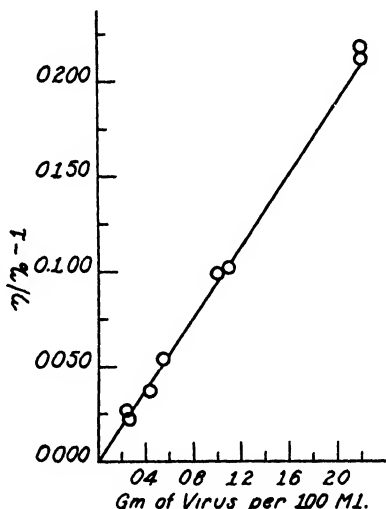


FIG. 3. Relationship between concentration and the specific viscosity of a preparation of Lee influenza virus.

however, practically no change, for it possessed an intrinsic viscosity of 9.44.

The relationship between concentration and the specific viscosity of the RBC preparation used in the sedimentation studies of Fig. 2 is shown graphically in Fig. 3. The intrinsic viscosity, calculated from the slope of the straight line drawn through the experimental points, was 9.50. The differences in sedimentation rates at varying concentrations of virus, shown by the data of Fig. 2, can be accounted for very closely by corresponding differences in relative viscosity provided by the data of Fig. 3. Multiplication of sedimentation rate and relative viscosity yields nearly constant values over the range of concentration of virus studied. This relationship is in accord with that previously established with other viruses (4, 23, 24). The sedimentation and viscosity data for the virus fractions of Fig. 1 do

not, however, yield constant results when dealt with as above, indicating effects of other, unknown factors in these instances.

If the Lee virus particles are assumed to be spherical, and the reported values of 0.863 ml. per gm. and 1.104 gm. per ml. are assumed for the anhydrous partial specific volume and the hydrated density, respectively, of the particles (22), the theoretical intrinsic viscosity, calculated from the Einstein equation (25), $\eta/\eta_0 - 1 = 2.50\phi$, where ϕ represents the volume fraction of the solute, becomes 3.46. The discrepancy between this value and the value of 9.50 reported above indicates the presence of small amounts of either highly viscous impurity or non-spherical virus structures such as the filamentous and branched forms reported by Mosley and Wyckoff (26). Similar calculations for the PR8 strain from available data (22) give a value

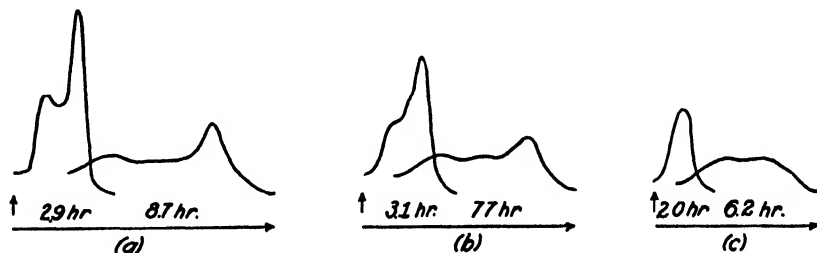


FIG. 4. Tracings of Longworth scanning diagrams of ascending boundaries of Lee influenza virus fractions. The arrows indicate the positions of starting boundaries. Intervals of time are based on a field strength of 1.35 volts per cm. (a) Crude unfractionated virus; mobilities, 0.33, 0.52, and 0.65×10^{-4} cm. per second per volt per cm., respectively. (b) Heavy centrifuge fraction; mobilities, 0.31, 0.47, and 0.65×10^{-4} cm. per second per volt per cm., respectively. (c) RBC preparation; mobilities, 0.35 and 0.52×10^{-4} cm. per second per volt per cm., respectively.

of 4.73 for the theoretical intrinsic viscosity, whereas the minimal experimental value reported has been 11.3 (4).

Electrophoretic Studies—Moving boundary electrophoretic diagrams obtained for crude, heavy, and RBC fractions of Lee virus preparations are shown in Fig. 4.¹ The light fractions were not studied by the moving boundary method. The crude virus exhibits three major components whose mobilities are 0.33, 0.52, and 0.65×10^{-4} cm. per second per volt per cm., respectively. The heavy fraction also reveals these same components. This indicates that the light material which was removed by centrifugal fractionation possessed an electrophoretic mobility approximately the same as that of one or more of the heavy components, and thus

¹ The sloping base lines, which may be noted in the electrophoretic diagrams of Fig. 4, were also characteristic of sedimentation diagrams, as shown in Fig. 1, and appeared to be due in some way to the high opalescence of the virus preparations.

its removal was not readily detectable. Diagrams for the RBC preparation, on the other hand, reveal only two major components, the more rapidly migrating one in the crude and heavy preparations having been removed. Other RBC preparations also showed only two components and differed from the preparation shown in Fig. 4 in exhibiting greater or lesser spread of the boundaries during electrophoretic migration.

It is evident from the data just described that the rapidly migrating component of the crude and heavy preparations represents an impurity and, further, that the apparent proportion of the impurity, as indicated by the areas under the peaks, is around 50 per cent. To substantiate this evidence, electrophoretic fractionation was carried out. A solution of crude virus containing 4 mg. of material per ml. was subjected to electrophoresis with mechanical compensation (27) for a period of time which, from the differences in rates of migration, should have placed the pure fast component in the uppermost compartment of the ascending limb of the U-tube and the slower components in the uppermost compartment of the descending limb. The slow and fast fractions thus obtained possessed chicken red blood cell agglutination titers of 2770 and 108 units per mg., respectively. Corresponding infectivity measurements in chick embryos yielded 50 per cent end-points at dilutions of 10^{-11} ²⁸ and 10^{-10} ¹⁵ gm. per ml. By the agglutination test, the slow fraction was, therefore, 26 times as active as the fast; by the infectivity measurements, 12 times as active. The results obtained by the two methods may be considered in fair agreement within their limits of accuracy. Because of the possibility of contamination of the fast fraction with the more slowly migrating material during electrophoresis (8), the rapidly migrating component may actually have been completely inactive.

Micro electrophoresis measurements yielded isoelectric points for the crude, light, heavy, and RBC preparations of 5.0, 4.6, 5.1, and 5.4, respectively. These results show that centrifugal fractionation increased the amount of acidic material in the light fraction and decreased the amount in the heavy fraction. The isoelectric point of the RBC preparation is much higher than that of the heavy fraction, indicating the presence of still less acidic material. This acidic material can nearly certainly be correlated with the most rapidly moving boundaries of the crude and heavy fractions shown in Fig. 4, the absence of which from the RBC preparation explains the higher isoelectric point observed in that case.

Curves of mobility *versus* pH obtained by the micro electrophoresis method for several crude and purified virus preparations are shown in Fig. 5. The lower mobilities of the crude virus on the acid side of the isoelectric point and higher mobilities on the alkaline side, when compared with corresponding mobilities of the purified virus, reflect the influence of the presence

in the former of an acidic impurity. The agreement in results obtained for electrophoretically purified virus with those for RBC virus indicates comparable degrees of purity for the two different types of preparations. It should be pointed out that, in view of the low degree of electrochemical homogeneity of Lee virus preparations as shown by the moving boundary diagrams of Fig. 4, the values of the mobilities shown in Fig. 5 may at best be considered only average, or approximate ones.

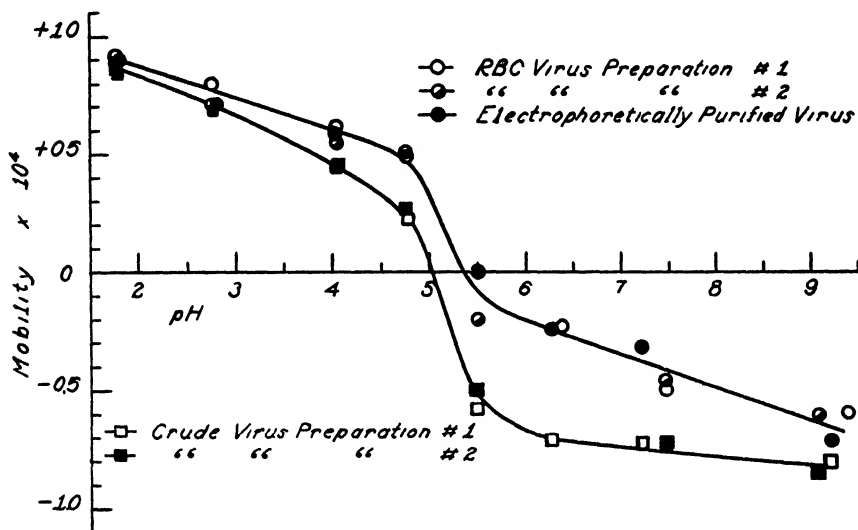


FIG. 5. Mobilities and isoelectric points of crude, RBC, and electrophoretically purified Lee influenza virus preparations

DISCUSSION

Crude Lee virus preparations appear to contain impurities whose over-all characteristics are a wide range in particle sizes, a high viscosity, and acidic electrophoretic properties. The data also show that both the small and the large particles of the impurity are inactive, viscous, and acidic. Except for the range in particle size, these properties correspond in general to those of the normal heavy material elaborated by uninfected embryos. If the large particles represent degradation products or precursors of the virus, they must be much more asymmetric and possess a higher proportion of acidic groupings than the virus itself.

Purified Lee virus preparations show a higher sedimentation constant than corresponding preparations of PR8 virus. This confirms findings of other workers (1, 2, 10, 28). It may be noted, however, that whereas crude Lee virus in the present studies and those of the Duke University investigators (10) appeared less homogeneous and less readily capable of purification

than PR8 virus, the PR8 strain was found the least homogeneous by Friedewald and Pickels (2). The possibility is therefore suggested that single strains of influenza virus may undergo mutation in different laboratories. Salk (29) has recently reported additional evidence of such changes.

The isoelectric point of purified preparations of Lee virus, namely about pH 5.4, was not significantly different from that of the PR8 strain. The Lee virus showed a differently shaped curve of mobility *versus* pH, however, and was found less homogeneous in moving boundary tests than the PR8 virus. The appearance of a double electrophoretic boundary for the Lee virus was an unusual finding, but seemed reproducible and characteristic of the strain under examination.

The average specific chicken red blood cell agglutinating activity of the purest Lee virus preparations, about 3000 units per mg. of virus, was significantly lower than maximum values in the neighborhood of 4000 units per mg. reported for the PR8 strain (4, 5, 8). Knight has already reported the same findings in agglutination measurements of RBC preparations of both strains (9) and so the distinction appears well established. The differences are of practical interest for purposes of characterization, although from a theoretical standpoint the arbitrary conditions of the test may favor the apparent activity of one strain over the other since, as has been shown by Hirst (16), the two viruses behave somewhat differently in their reactions with red cells.

SUMMARY

Crude Lee virus preparations obtained by high speed centrifugation were found to contain 30 to 50 per cent of impurity, based on measurements of chicken red blood cell agglutinating activity and electrophoretic studies. The impurity was characterized by a wide range of particle sizes, high viscosity, and acidic electrophoretic properties. Removal of small sized particles of impurity was effected by fractional centrifugation, but electrophoretic fractionation or adsorption of the virus on chicken red blood cells and elution therefrom was required to remove both large and small particles of impurity. Large particles of the impurity were similar to the virus in sedimentation rate but more like the normal heavy material elaborated by uninfected embryos in viscosity and electrophoretic properties.

Purified Lee virus preparations exhibited a single sedimentable component representing a family of particles with an average sedimentation constant of 802 Svedberg units at infinite dilution and a standard deviation of 6.7 per cent from the mean sedimentation rate. The intrinsic viscosity was found to be 9.50 compared with the theoretical value of 3.46 for spherical particles calculated from available data with the aid of the Einstein equation, suggesting the presence of small amounts of either highly viscous im-

purity or non-spherical virus structures. Moving boundary experiments revealed the presence of two very diffuse boundaries, both appearing, however, to represent the virus. The isoelectric point, determined by the micro electrophoresis method, was at approximately pH 5.4. The chicken red blood cell agglutinating activity was 3000 units per mg., a value significantly less than that of about 4000 units per mg. reported previously for the PR8 strain.

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LETTERS TO THE EDITORS

THE DEMONSTRATION OF THE OXIDATION IN VIVO OF THE METHYL GROUP OF METHIONINE*

Sirs:

The recent synthesis¹ in this laboratory of methionine containing C¹⁴ in the methyl radical has enabled us to demonstrate for the first time the oxidation of the labile methyl group fed in the form of this amino acid. Furthermore, the synthesis of radiomethyl methionine has made possible a balance study on the distribution of its methyl carbon in the organs and tissues of the body, the expired air, and the urine and feces.

In the present experiment, 200 mg. of L-methionine containing C¹⁴ in the methyl group were given by stomach tube to a 165 gm. rat. The animal was immediately placed in an open circuit metabolism apparatus, and the expired carbon dioxide was collected continuously in sodium hydroxide solution. The carbon dioxide was precipitated at intervals as barium carbonate, and its radioactivity determined by means of a thin mica window Geiger-Müller counter.

The presence of radioactivity in the carbon dioxide collected even in the 1st hour of the experiment furnished conclusive proof that the labile methyl group, when ingested as methionine, can be oxidized to carbon dioxide. At the end of 12 hours, 17 per cent of the administered methyl carbon had appeared in this form. 52 hours after the start of the experiment we were able to account for 32.4 per cent of the methyl carbon of the administered methionine in the expired carbon dioxide. During this time 14.6 per cent of the methyl carbon was excreted in the urine and 0.4 per cent in the feces.

The rat was sacrificed at 52 hours, and separate samples of its tissues and organs were burned. The carbon dioxide evolved in each combustion was recovered as barium carbonate. Radioactivity determinations indicated that the methyl carbon had been most highly concentrated in the kidneys, liver, and adrenals in the order named. Intermediate concentrations were found in the intestines, pancreas, and testes, while smaller concentrations were found in the blood, skin, muscle, and brain.

* The authors wish to express their appreciation to the Lederle Laboratories Division, American Cyanamid Company, for a grant-in-aid which has helped to make this work possible.

¹ Melville, D. B., Rachele, J. R., and Keller, E. B., *J. Biol. Chem.*, **169**, 419 (1947).

Estimation, on the basis of the tissue analyses, of the portion of the originally introduced methyl carbon still present in the rat's body, and the addition of this sum to the amounts found in the expired carbon dioxide, the urine, and the feces, revealed that the recovery of the methyl carbon originally supplied as methionine was almost quantitative.

The availability of C^{14} methyl iodide¹ together with the methods developed in the course of the present study has permitted us to initiate comparable investigations of the oxidation and distribution of the methyl carbon of creatine, choline, and other methyl compounds. Now that the oxidation of the labile methyl of methionine is established, the intermediary products in its conversion to carbon dioxide and the determination of their rôles in metabolic processes become matters of considerable interest.

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METABOLIC CONNECTION BETWEEN PROLINE AND GLYCINE IN THE AMINO ACID UTILIZATION OF *TORULOPSIS UTILIS*

Sirs:

DL-Alanine and glycine, the carboxyl groups of which contained an excess of C¹³, have been given to *Torulopsis* yeast as a single carbon source.¹ After 120 minutes the yeast was washed and hydrolyzed; arginine, lysine, glycine, glutamic acid, and aspartic acid were isolated in pure form² and fractions containing alanine, serine, and proline were prepared. These were treated with ninhydrin³ and the CO₂ liberated was analyzed in the mass spectrograph. The results are recorded in the table.

Amino acids given to <i>Torulopsis</i> yeast	Per cent excess C ¹³ in α-carboxyl group	
	2.17	2.22
DL-Alanine . .		
Glycine		
Amino acids isolated from hydrolysate		
Aspartic acid (both carboxyl groups)	0.12	0.08
Glutamic acid	0.09	0.08
Arginine	0.14	
Lysine		0.04
Alanine fraction (+ small amounts of serine)	0.25	
Leucine-phenylalanine fraction	0.12	
Valine fraction (+ small amounts of proline)	0.07	
Glycine (nitranilic acid removed)		0.54
Serine fraction (+ small amounts of alanine)		0.46
Proline fraction		0.29
Whole yeast (total carbon) calculated	0.03	0.03
Respiratory CO ₂ liberated during		
0- 45 min.	0.58	0.32
45- 90 " .	0.57	0.53
90-120 " .	0.41	0.62

In the alanine experiment the isotope appeared to be rather evenly distributed among the various amino acids, and showed a marked excess only in alanine itself. In the glycine experiment the marked carboxyl seems to have been preponderantly transferred to the serine and proline

¹ Sperber, E., *Ark. Kemi, Mineral. o. Geol.*, **21 A**, No. 3, 72 (1945).

² Sperber, E., *J. Biol. Chem.*, **166**, 75 (1946). Town, B. W., *Biochem. J.*, **30**, 1837 (1936).

³ Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, **150**, 251 (1943).

fractions. This is interesting in view of the results of Shemin,⁴ who has demonstrated in mammals a definite conversion of serine into glycine. Our findings indicate a reversal of this effect. The formation of proline from glycine, indicated by our results, may occur in a way analogous to that suggested by Shemin and Rittenberg⁵ for the formation of porphyrins.

As to the over-all distribution of the α -carboxyl C¹³, the mechanism of this reaction is still obscure. One explanation of this apparent "trans-carboxylation" might be that a carboxylation from a common pool of fixed CO₂ is involved in the synthesis of peptide linkages, and thus the excess C¹³ in the respiratory CO₂ will in part appear as carboxyl groups of the amino acid residues. The experiments of Evans and Slotin⁶ with radioactive CO₂, which after fixation could in part be released with ninhydrin, also point in this direction.

The mass spectrometric measurements were carried out by Dr. E. Lindholm, to whom we express our thanks. The accuracy of the measurements is of the order 5:1000.

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⁴ Shemin, D., *J. Biol. Chem.*, **162**, 297 (1946).

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⁶ Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **141**, 439 (1941).

THE BIOTIN ACTIVITY OF A VACCENIC ACID FRACTION

Sirs:

The recent demonstrations of the interrelationship between biotin and various lipoidal substances in microbial growth are significant in their implications regarding the mode of action of biotin. Thus, oleic and elaidic acids¹ and a non-saponifiable, ether-soluble substance from hydrolyzed plasma² have been shown to replace biotin in the nutrition of *Lactobacillus casei*. We have also prepared an ether-soluble, neutral material with biotin-like activity from plasma, but, in contrast to the latter report, have found the activity to be present in the saponifiable portion.³ Work is in progress to elucidate the nature of the active fatty acid(s) in this

Biotin-Like Activity of Various Fatty Acids

Substance	Activity, as μ g biotin per mg. substance, for			
	Hemolytic streptococcus	<i>L. arabinosus</i>	<i>L. casei</i>	<i>S. cerevisiae</i>
Oleic acid .	<0.05	5.0	11.5	0.6
Elaidic acid .	<0.05	1.0	15.2	<0.05
Vaccenic acid fraction	<0.05	1.2	5.3	<0.05

fraction. The recent report of Boer *et al.*⁴ attributing the growth-promoting properties of summer butter for rats to a vaccenic acid fraction stimulated our interest in the possible microbiological activity of this fraction.

The vaccenic acid fraction (iodine number 84.5, m.p. 38°) was obtained from beef tallow by a modification of the procedure employed by Bertram.⁵ The biotin-like activity of this fraction, as well as that of oleic and elaidic acids, was determined for the following biotin-requiring organisms: a strain of hemolytic streptococcus group C, *Lactobacillus arabinosus*, *Lactobacillus casei*, and *Saccharomyces cerevisiae* 139. Highly purified basal media which are adequate for growth when supplemented with biotin were employed in these studies. The relative effectiveness of the various fatty acids in stimulating growth under these conditions is shown in the table.

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³ Hofmann, K., and Axelrod, A. E., unpublished observations.

⁴ Boer, J., Jansen, B. C. P., and Kentie, A., *J. Nutr.*, **33**, 339 (1947).

⁵ Bertram, S. H., *Biochem Z.*, **197**, 433 (1928).

It is evident that these fatty acids, although inactive for hemolytic streptococcus and *S. cerevisiae* (with the exception of oleic acid), are capable of stimulating the growth of *L. arabinosus* and *L. casei*. The microbiological activity of the vaccenic acid fraction is of interest in view of its growth-promoting activity for the rat. As contrasted with vaccenic acid, neither oleic acid nor elaidic acid has been shown to possess any specific activity for higher animals.

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PEPTIDE SYNTHESIS IN VIVO*

Sirs:

The mechanism of protein synthesis is not known. It may be postulated that amino acids are united to form peptides, which in turn condense to form proteins. However, the observed rapid rates of incorporation of labeled amino acids into tissue proteins throw doubt upon the intermediary rôle of peptides in protein synthesis.

The experiment reported here demonstrates the formation of a C¹⁴-labeled dipeptide, leucylglycine*, following the administration of a mixture of carboxyl-labeled radioactive glycine (glycine*) and ordinary L-leucine to rats. Ordinarily this peptide might be utilized so rapidly as to make its isolation impossible. Hence it was trapped by the injection of leucylglycine prior to sacrifice of the animals. Subsequently the leucylglycine* was isolated from tissue extracts with the aid of additional carrier, and purified to constant specific radioactivity.

Two 180 gm. rats each received 25 mg. of glycine* (20,000 counts per mg. per minute) and 40 mg. of L-leucine by way of the jugular vein. 10 minutes later 60 mg. of leucylglycine were given by the same route. After 6 more minutes the animals were sacrificed. (Other experiments showed that such a time interval does not permit the incorporation of significant amounts of glycine* into proteins.) The liver, kidney, spleen, lung, and intestine of both animals were collectively homogenized with 10 per cent trichloroacetic acid. The non-protein filtrate of these organs contained 70,000 counts per minute (about 7 per cent of the administered glycine*). To this solution was added 1 gm. of leucylglycine. Then the trichloroacetic acid was removed by extraction with ether and the solution was concentrated and adjusted to pH 5.8. The peptide was precipitated by the addition of 2 to 3 volumes of acetone, and recrystallized six times by dissolving it in hot water, and then adding acetone.

The specific radioactivity of the recovered leucylglycine* remained constant during the last three recrystallizations. The final product (185 mg.) gave 8.0 counts per mg. per minute. Since 1 gm. of carrier had been added, the leucylglycine* would account for 8000 out of 70,000 counts or about 11 per cent of the total radioactivity of the trichloroacetic acid filtrate. Evidently the experimental conditions chosen favored the preferential synthesis of the peptide. It may be mentioned that when similar

* Aided by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council) and the Rockefeller Foundation;

experiments were conducted with tissue homogenates the isolated leucylglycine contained no detectable radioactivity.

The following tests indicate that the isolated leucylglycine* did not contain free glycine: (1) No radioactive CO_2 was obtained upon treatment of the peptide with ninhydrin. Following acid hydrolysis of the peptide, radioactive CO_2 was liberated by ninhydrin. (2) When glycine* was added to either labeled (recovered) leucylglycine or to ordinary leucylglycine, 97 to 99 per cent of the glycine could be recovered by two recrystallizations.

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FELIX FRIEDBERG
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Received for publication, July 7, 1947

- Fishman, William H.** β -Glucuronidase: its relation to the action of the estrogenic hormones, 7
- and **Anlyan, A. J.** The presence of high β -glucuronidase activity in cancer tissue, 449
- Flemister, Sarah C.** See *Scholander, Flemister, and Irving*, 173
- Flinn, Barbara C.** See *Azelrod, Flinn, and Hofmann*, 195
- Floyd, Curtis S.** See *Gjessing and Chanutin*, 657
- Folch, Jordi, and Lauren, Miriam.** Estimation of potassium in biological materials as potassium phosphotungstate, 539
- Franklin, A. L., Li, Choh Hao, and Dunn, Max S.** The amino acid composition of anterior hypophyseal growth hormone, 515
- , **Stokstad, E. L. R., Belt, Margaret, and Jukes, Thomas H.** Biochemical experiments with a synthetic preparation having an action antagonistic to that of pteroylglutamic acid, 127
- Frantz, Ivan D., Jr., and Stephenson, Mary L.** A study of the reaction kinetics of a catheptic enzyme with a decarboxylase as indicator, 359
- Friedberg, Felix, Winnick, Theodore, and Greenberg, David M.** Peptide synthesis *in vivo*, 763
- Friedkin, Morris, and Lehninger, Albert L.** The synthesis of phosphomalic acid, 183
- Fruton, Joseph S., Simmonds, Sofia, and Smith, Virginia A.** The action of *Escherichia coli* on acetyldehydro-amino acids, 267
- See *Simmonds, Tatum, and Fruton*, 91
- See *Yudkin and Fruton*, 521

G

- Galston, Arthur W.** Synergism between indoleacetic and nicotinic acids in a plant growth inhibition, 465
- Geyer, R. P., Nath, H., Barki, V. H., Elvehjem, C. A., and Hart, E. B.** The vaccenic acid content of various fats and oils, 227
- Gjessing, Erland C., and Chanutin, Alfred.** An electrophoretic study of plasma and plasma fractions of normal and injured rats, 657
- Goldwater, William H., and Stetten, DeWitt, Jr.** Studies in fetal metabolism, 723
- Gollub, Miriam C., and Vennesland, Birgit.** Fixation of carbon dioxide by a plant oxalacetate carboxylase, 233
- Gonçalves, Jose M., Price, Vincent E., and Greenstein, Jesse P.** Enzymatic hydrolysis of acetyldehydroalanine, 225
- Gowen, Leo F. X.** See *Michel and Scheinberg*, 277
- Gray, Charles H., and Holt, Lewis B.** The porphyrin produced by diphtheria bacillus, 235
- Greenberg, David M.** See *Friedberg, Winnick, and Greenberg*, 763
- Greenstein, Jesse P.** See *Gonçalves, Price, and Greenstein*, 225
- Guest, M. Mason.** See *Ware, Guest, and Seegers*, 231
- Gurin, Samuel.** See *Buchanan, Sakami, and Guin*, 411
- See *Buchanan, Sakami, Gurin, and Wilson*, 403
- Gustavson, K. H.** Note on the reaction of formaldehyde with collagen, 531
- Gutmann, Helmut R., Jandorf, Bernard J., and Bodansky, Oscar.** The rôle of pyridine nucleotides in the reduction of methemoglobin, 145

H

- Hack, M. H.** Estimation of the phospholipides in human blood, 137
- Haines, William J.** See *Carter, Haines, Ledyard, and Norris*, 77
- Hanahan, Donald J., and Chalkoff, I. L.** A new phospholipide-splitting enzyme specific for the ester linkage between the nitrogenous base and the phosphoric acid grouping, 699

- Hariton, L.** See *Dobriner, Lieberman, Hariton, Sarett, and Rhoads*, 221
- Hart, E. B.** See *Geyer, Nath, Barki, Elvehjem, and Hart*, 227
- Henriques, F. C., Jr.** See *DeMeio and Henriques*, 609
- Heuser, G. F.** See *Daniel, Norris, Scott, and Heuser*, 689
- Hodge, Harold Carpenter, MacLachlan, P. L., Bloor, W. R., Welch, Eileen A., Kornberg, Sylvia Levy, and Falkenheim, Marlene.** Lipides of the fasting mouse. III. Phospholipide to neutral fat relationship in the blood lipides, 707
- See *Falkenheim, Neuman, and Hodge*, 713
- Hofmann, Klaus, Winnick, Theodore, and Axelrod, A. E.** The use of Rancy's nickel in a differential assay for oxybiotin and biotin, 191
- See *Axelrod, Flinn, and Hofmann*, 195
- See *Axelrod, Hofmann, and Daubert*, 761
- Hogness, Katharine R.** See *Petermann, Young, and Hogness*, 379
- Holt, Lewis B.** See *Gray and Holt*, 235
- Horn, Millard J., Jones, D. Breese, and Blum, Amos E.** Microbiological determination of lysine in proteins and foods, 71
- , —, and —. Microbiological determination of threonine in proteins and foods, 739
- Huebner, Charles F., and Jacobs, Walter A.** The aconite alkaloids. XVI. On staphisine and the hydrocarbon obtained from its dehydrogenation, 211
- Huffman, Max N.** 16-Substituted steroids. III. The partial synthesis of estriol, 167
- Hutchings, B. L., and Boggiano, E.** Oleic acid as a growth factor for various lactobacilli, 229

I

- Irving, Laurence.** See *Scholander, Flemister, and Irving*, 173
- See *Scholander and Irving*, 561

J

- Jacobs, Walter A.** See *Huebner and Jacobs*, 211
- Jaffé, Werner G., and Elvehjem, C. A.** Fractionation of growth-stimulating factor in liver, 287
- Jandorf, Bernard J.** See *Gutmann, Jandorf, and Bodansky*, 145
- Jelinek, Viola C.** See *Boxer, Jelinek, and Leghorn*, 153
- Jervis, George A.** Studies on phenylpyruvic oligophrenia. The position of the metabolic error, 651
- Johnson, R. M., and Baumann, C. A.** Studies on the reaction of certain carotenoids with antimony trichloride, 83
- Jones, D. Breese.** See *Horn, Jones, and Blum*, 71, 739
- Jukes, Thomas H.** See *Franklin, Stokstad, Belt, and Jukes*, 427

K

- Kalman, C.** See *Li, Kalman, and Evans*, 625
- Keller, Elizabeth B.** See *Mackenzie, Chandler, Keller, Rachele, Cross, Melville, and du Vigneaud*, 757
- See *Melville, Rachele, and Keller*, 419
- Kochakian, Charles D., and Vail, Virginia N.** The effect of adrenalectomy, adrenal cortical hormones, and testosterone propionate plus adrenal cortical extract on the arginase activity of the liver and kidney of the rat, 1
- Kornberg, Sylvia Levy.** See *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707

L

- Lamanna, Carl.** See *Buehler, Schantz, and Lamanna*, 295
- Lardy, Henry A., Potter, Richard L., and Elvehjem, C. A.** The rôle of biotin in bicarbonate utilization by bacteria, 451
- Lauren, Miriam.** See *Folch and Lauren*, 539
- Lavine, Theodore F.** The formation, resolution, and optical properties of

- the diastereoisomeric sulfoxides derived from L-methionine, 477
- Ledyard, W. E.** See *Carter, Haines, Ledyard, and Norris*, 77
- Leghorn, Patricia M.** See *Boxer, Jelinek, and Leghorn*, 153
- Lehninger, Albert L.** See *Friedkin and Lehninger*, 183
- Lens, J.** The inactivation of insulin solutions, 313
- Lewis, Howard B.** See *Schofield and Lewis*, 373
- Li, Choh Hao, Kalman, C., and Evans, Herbert M.** The effect of the hypophyseal growth hormone on the alkaline phosphatase of rat plasma, 625
- See *Franklin, Li, and Dunn*, 515
- See *Mason, Power, Rynearson, Ciaramelli, Li, and Evans*, 223
- Lieberman, Seymour.** See *Dobriner, Lieberman, Hariton, Sarett, and Rhoads*, 221
- Loring, Hubert S., Carpenter, F. H., and Roll, Paul M.** The hydrolysis of yeast ribonucleic acid by ribonucleinase. I. The extent of hydrolysis and the preparation of ribonucleinase-resistant fractions after ribonucleinase treatment, 601

M

- Mackenzie, Cosmo G., Chandler, Joseph P., Keller, Elizabeth B., Rachele, Julian R., Cross, Nancy, Melville, Donald B., and du Vigneaud, Vincent.** The demonstration of the oxidation *in vivo* of the methyl group of methionine, 757
- MacLachlan, P. L.** See *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707
- Major, C. L. H.** The possibility of detecting individual proteins in blood serum by differentiation of solubility curves in concentrated sodium sulfate solutions. II. Comparison of solubility curves with results of electrophoresis experiments, 583
- Martin, Gustav J.** See *Beiler and Martin*, 345
- Mason, Harold L., Power, Marschelle H., Rynearson, E. H., Ciaramelli, L. C., Li, Choh Hao, and Evans, Herbert M.** Results of administration of anterior pituitary adrenocorticotrophic hormone to a normal human being, 223
- McGilvery, R. W.** See *Cohen and McGilvery*, 119
- Melville, Donald B., Rachele, Julian R., and Keller, Elizabeth B.** A synthesis of methionine containing radiocarbon in the methyl group, 419
- See *Mackenzie, Chandler, Keller, Rachele, Cross, Melville, and du Vigneaud*, 757
- Meyerhof, Otto.** See *Polis and Meyerhof*, 389
- Michel, Harry O., and Scheinberg, I. Herbert.** The effects of cytochrome c on anoxic and cyanide-poisoned rats, 277
- Miller, Gail Lorenz.** Sedimentation, viscosity, and electrophoretic studies on purified Lee influenza virus preparations, 745
- Milne, John.** Serum protein fractionation: a comparison of sodium sulfate precipitation and electrophoresis, 595
- Morgan, J. F., and Campbell, M. E.** A rapid method for the production and isolation of penicillinase, 337
- Morrison, Peter R.** An automatic apparatus for the determination of oxygen consumption, 667

N

- Nath, H.** See *Geyer, Nath, Barki, Elvehjem, and Hart*, 227
- Neuman, William F.** See *Falkenheim, Neuman, and Hodge*, 713
- Norris, L. C.** See *Daniel, Norris, Scott, and Heuser*, 689
- Norris, William P.** See *Carter, Haines, Ledyard, and Norris*, 77

O

- Olson, Kenneth J., Ely, Ray E., and Reineke, E. P.** An improved method for the determination of thiouracil, 681

P

- Petermann, Mary L., Young, Nelson F., and Hogness, Katharine R.** A comparison of the Howe and the electrophoretic methods for the determination of plasma albumin, 379
- Polis, B. David, and Meyerhof, Otto.** Studies on adenosinetriphosphatase in muscle. I. Concentration of the enzyme on myosin, 389
- Portis, Richard A., and Altman, Kurt I.** The action of proteolytic enzymes and protaminase on salmine sulfate, 203
- Potter, Richard L.** See *Lardy, Potter, and Elvehjem*, 451
- Potter, Van R.** The assay of animal tissues for respiratory enzymes. VI. Further studies on oxidative phosphorylation, 17
- Power, Marschelle H.** See *Mason, Power, Rynearson, Ciaramelli, Li, and Evans*, 223
- Price, Vincent E.** See *Gonçalves, Price, and Greenstein*, 225

Q

- Quaife, Mary Louise.** Tocopherols (vitamin E) in milk: their chemical determination and occurrence in human milk, 513

R

- Rabinowitz, Jesse C., and Snell, Esmond E.** The vitamin B₆ group. XI. An improved method for assay of vitamin B₆ with *Streptococcus faecalis*, 631
- and —. XII. Microbiological activity and natural occurrence of pyridoxamine phosphate, 643
- Rachele, Julian R.** See *Mackenzie, Chandler, Keller, Rachele, Cross, Melville, and du Vigneaud*, 757
- See *Melville, Rachele, and Keller*, 419
- Reineke, E. P.** See *Olson, Ely, and Reineke*, 681
- Reio, L.** See *Ehrensward, Sperber, Saluste, Reio, and Stjernholm* 759
- Rhoads, C. P.** See *Dobriner, Lieberman, Hariton, Sarett, and Rhoads*, 221
- Rhymer, Ione, Wallace, G. I., Byers, L. W., and Carter, Herbert E.** The antistreptomycin activity of lipositol, 457
- Ringel, S. J.** See *Csonka, Denton, and Ringel*, 259
- Rittenberg, D.** See *Bloch and Rittenberg*, 467
- Rogers, Lorene Lane, and Shive, William.** Biochemical transformations as determined by competitive analogue-metabolite growth inhibitions. VI. Prevention of biotin synthesis by 2-oxo-4-imidazolidinecaproic acid, 57
- See *Shive and Rogers*, 453
- Roll, Paul M.** See *Loring, Carpenter, and Roll*, 601
- Rynearson, E. H.** See *Mason, Power, Rynearson, Ciaramelli, Li, and Evans*, 223

S

- Sakami, Warwick.** See *Buchanan, Sakami, and Gurin*, 411
- See *Buchanan, Sakami, Gurin, and Wilson*, 403
- Saluste, E.** See *Ehrensward, Sperber, Saluste, Reio, and Stjernholm*, 759
- Sarett, Lewis Hastings.** See *Dobriner, Lieberman, Hariton, Sarett, and Rhoads*, 221
- Schantz, E. J.** See *Buehler, Schantz, and Lamanna*, 295
- Scheinberg, I. Herbert.** See *Michel and Scheinberg*, 277
- Schofield, Frances A., and Lewis, Howard B.** A comparative study of the metabolism of α -alanine, β -alanine, serine, and isoserine. II. Glycogen content of the liver after oral administration of the amino acids, 373
- Scholander, P. F., and Evans, H. J.** Microanalysis of fractions of a cubic millimeter of gas, 551
- , **Flemister, Sarah C., and Irving, Laurence.** Microgasometric estimation of the blood gases. V. Combined carbon dioxide and oxygen, 173
- and **Irving, Laurence.** Micro blood gas analysis in fractions of a cubic millimeter of blood, 561

- Scott, M. L.** See *Daniel, Norris, Scott, and Heuser*, 689
- Seegers, Walter H.** See *Ware, Guest, and Seegers*, 231
- Shive, William, and Rogers, Lorene Lane.** Involvement of biotin in the biosynthesis of oxalacetic and α -ketoglutaric acids, 453
— See *Rogers and Shive*, 57
- Shorb, Mary S.** Unidentified growth factors for *Lactobacillus lactis* in refined liver extracts, 455
- Shorr, Ephraim.** See *Taussky and Shorr*, 103
- Simmonds, Sofia, Tatum, E. L., and Fruton, Joseph S.** The utilization of phenylalanine and tyrosine derivatives by mutant strains of *Escherichia coli*, 91
— See *Fruton, Simmonds, and Smith*, 267
- Simonsen, Daisy G., Westover, Leola M., and Wertman, Maxine.** The determination of serum magnesium by the molybdivanadate method for phosphate, 39
- Sizer, Irwin W.** The action of tyrosinase on certain proteins and products of their autolysis, 303
- Smith, Virginia A.** See *Fruton, Simmonds, and Smith*, 267
- Snell, Esmond E.** See *Rabinowitz and Snell*, 631, 643
- Sperber, E.** See *Ehrensward, Sperber, Saluste, Reio, and Stjernholm*, 759
- Stephenson, Mary L.** See *Frantz and Stephenson*, 359
— See *Zamecnik and Stephenson*, 349
- Stetten, DeWitt, Jr.** See *Goldwater and Stetten*, 723
- Stjernholm, R.** See *Ehrensward, Sperber, Saluste, Reio, and Stjernholm*, 759
- Stokstad, E. L. R.** See *Franklin, Stokstad, Belt, and Jukes*, 427
- Struglia, Lena.** See *Daniel, Norris, Scott, and Heuser*, 689
- Stumpf, P. K.** A colorimetric method for the determination of desoxyribonucleic acid, 367

T

- Tatum, E. L.** See *Simmonds, Tatum, and Fruton*, 91
- Taugog, Alvin, and Chaikoff, I. L.** The metabolic interrelations of thyroxine and diiodotyrosine in the thyroid gland as shown by a study of their specific activity-time relations in rats injected with radioactive iodine, 49
- Taussky, Hertha H., and Shorr, Ephraim.** A microcolorimetric method for the determination of citric acid, 103

V

- Vail, Virginia N.** See *Kochakian and Vail*, 1
- Van Slyke, Donald D.** See *Chinard and Van Slyke*, 571
- Vennesland, Birgit.** See *Gollub and Vennesland*, 233
- Vickery, Hubert Bradford.** Rules for the nomenclature of the natural amino acids and related substances, 237
- du Vigneaud, Vincent.** See *Dittmer and du Vigneaud*, 63
— See *Mackenzie, Chandler, Keller, Rachele, Cross, Melville, and du Vigneaud*, 757

W

- Wallace, G. I.** See *Rhymer, Wallace, Byers, and Carter*, 457
- Ware, Arnold G., Guest, M. Mason, and Seegers, Walter H.** A factor in plasma which accelerates the activation of prothrombin, 231
- Welch, Eileen A.** See *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707
- Wertman, Maxine.** See *Simonsen, Westover, and Wertman*, 39
- Westover, Leola M.** See *Simonsen, Westover, and Wertman*, 39
- Wilson, D. Wright.** See *Buchanan, Sakami, Gurin, and Wilson*, 403
- Winnick, Theodore.** See *Friedberg, Winnick, and Greenberg*, 763
— See *Hofmann, Winnick, and Axelrod*, 191

Y

- Young, Nelson F. See *Petermann, Young, and Hogness*, 379
Yudkin, Warren H., and Fruton, Joseph
-. On the proteolytic enzymes of animal tissues. VI. Dehydropeptidase, 521

Z

- Zamecnik, Paul C., and Stephenson, Mary L. A manometric method for determining the kinetics of an enzymatic hydrolysis of peptides, 349
Zvirblis, Peter. See *Michel and Scheinberg*, 277

INDEX TO AUTHORS

A

- Altman, Kurt I., and Evans, E. A., Jr.** Enzymic synthesis of triphosphopyridine nucleotide in cell-free extracts, 463
 —. See *Portis and Altman*, 203
Ames, Stanley R. Effect of calcium on the inhibition of the succinic oxidase system by *d*- α -tocopheryl phosphate, 503
Anker, H. S. See *Bloch and Anker*, 765
Anlyan, A. J. See *Fishman and Anlyan*, 449
Aronoff, S., Barker, H. A., and Calvin, M. Distribution of labeled carbon in sugar from barley, 459
Axelrod, A. E., Flinn, Barbara C., and Hofmann, Klaus. The metabolism of oxybiotin in yeast, 195
 —, **Hofmann, Klaus,** and **Daubert, B. F.** The biotin activity of a vaccenic acid fraction, 761
 —. See *Hofmann, Winnick, and Axelrod*, 191

B

- Barker, H. A.** See *Aronoff, Barker, and Calvin*, 459
Barki, V. H. See *Geyer, Nath, Barki, Elvehjem, and Hart*, 227
Baumann, C. A. See *Johnson and Baumann*, 83
Beiler, J. M., and Martin, Gustav J. Inhibition of the action of tyrosine decarboxylase by phosphorylated desoxypyridoxine, 345
Belt, Margaret. See *Franklin, Stokstad, Belt, and Jukes*, 427
Bird, Robert M. Glycolysis in human blood, 493
Bloch, Konrad, and Anker, H. S. Synthesis of glutathione in isolated liver, 765
 — and **Rittenberg, D.** The metabolism of acetyl amino acids, 467
Bloor, W. R. See *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707

- Blum, Amos E.** See *Horn, Jones, and Blum*, 71, 739
Bodansky, Oscar. See *Gutmann, Jan-dorf, and Bodansky*, 145
Boggiano, E. See *Hutchings and Boggiano*, 229
Bolomey, René A. Oxidative decomposition of vitamin A. I. Stability of vitamin A towards oxidation and irradiation, 323
 —. II. Absorption spectrophotometry of oxidized vitamin A, 331
Borsook, Henry, and Dubnoff, Jacob W. Methionine formation by transmethylation *in vitro*, 247
 — and —. Ornithine-catalyzed urea formation in liver homogenate, 461
Boxer, George E., Jelinek, Viola C., and Leghorn, Patricia M. The colorimetric determination of streptomycin in clinical preparations, urine, and broth, 153
Buchanan, John M., Sakami, Warwick, and Gurin, Samuel. A study of the mechanism of fatty acid oxidation with isotopic acetoacetate, 411
 —, —, —, and **Wilson, D. Wright.** Intermediates in the biological oxidation of isotopic acetoacetate, 403
Buehler, Henry J., Schantz, E. J., and Lamanna, Carl. The elemental and amino acid composition of crystalline *Clostridium botulinum* type A toxin, 295
Byers, L. W. See *Rhymer, Wallace, Byers, and Carter*, 457

C

- Calvin, M.** See *Aronoff, Barker, and Calvin*, 459
Campbell, M. E. See *Morgan and Campbell*, 337
Carpenter, F. H. See *Loring, Carpenter, and Roll*, 601
Carter, Herbert E., Haines, William J., Ledyard, W. E., and Norris, William P. Biochemistry of the sphingolipides. I. Preparation of sphingolip-

- ides from beef brain and spinal cord, 77
- Carter, Herbert E.** See *Rhymer, Wallace, Byers, and Carter*, 457
- Chaikoff, I. L.** See *Hanahan and Chaikoff*, 699
- See *Taurog and Chaikoff*, 49
- Chandler, Joseph P.** See *Mackenzie, Chandler, Keller, Rachele, Cross, Melville, and du Vigneaud*, 757
- Chanutin, Alfred.** See *Gjessing and Chanutin*, 657
- Chinard, Francis P., and Van Slyke, Donald D.** Comparison of a modified Folin photometric procedure and the ninhydrin manometric method for the determination of amino acid nitrogen in plasma, 571
- Ciaramelli, L. C.** See *Mason, Power, Rynearson, Ciaramelli, Li, and Evans*, 223
- Cohen, Philip P., and McGilvery, R. W.** Peptide bond synthesis. II. The formation of *p*-aminohippuric acid by liver homogenates, 119
- Cross, Nancy.** See *Mackenzie, Chandler, Keller, Rachele, Cross, Melville, and du Vigneaud*, 757
- Csonka, Frank A., Denton, Charles A., and Ringel, S. J.** The methionine and cystine content of hen's eggs, 259

D

- Daniel, Louise J., Norris, L. C., Scott, M. L., and Heuser, G. F.** Growth inhibition of bacteria by synthetic pterins. I Studies with *Streptococcus faecalis*, *Lactobacillus casei*, and *Lactobacillus arabinosus*, 689
- Daubert, B. F.** See *Archrod, Hofmann, and Daubert*, 761
- Day, Priscilla.** See *Michel and Scheinberg*, 277
- DeMeio, R. H., and Henriques, F. C., Jr.** Tellurium. IV. Excretion and distribution in tissues studied with a radioactive isotope, 609
- Denton, Charles A.** See *Csonka, Denton, and Ringel*, 259
- Deutsch, H. F.** A study of whey proteins from the milk of various animals, 437
- Dittmer, Karl, and du Vigneaud, Vincent.** Antibiotin activity of imidazolidone aliphatic acids, 63
- Dobriner, Konrad, Lieberman, Seymour, Hariton, L., Sarett, Lewis Hastings, and Rhoads, C. P.** The isolation of Δ^9 -etiocholesterol-3(α)-one-17 from human urine, 221
- Dubnoff, Jacob W.** See *Borsook and Dubnoff*, 247, 461
- Dunn, Max S.** See *Franklin, Li, and Dunn*, 515

E

- Ehrensward, G., Sperber, E., Saluste, E., Reio, L., and Stjernholm, R.** Metabolic connection between proline and glycine in the amino acid utilization of *Torulopsis utilis*, 759
- Elvehjem, C. A.** See *Geyer, Vath, Barki, Elvehjem, and Hart*, 227
- See *Jaffé and Elvehjem*, 287
- See *Lardy, Potter, and Elvehjem*, 451
- Ely, Ray E.** See *Olson, Ely, and Reineke*, 681
- Evans, E. A., Jr.** See *Altman and Evans*, 163
- Evans, H. J.** See *Scholander and Evans*, 551
- Evans, Herbert M.** See *Li, Kalman, and Evans*, 625
- See *Mason, Power, Rynearson, Ciaramelli, Li, and Evans*, 223

F

- Falkenheim, Marlene, Neuman, William F., and Hodge, Harold Carpenter.** Phosphate exchange as the mechanism for adsorption of the radioactive isotope by the calcified tissues, 713
- See *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707

SYNTHESIS OF GLUTATHIONE IN ISOLATED LIVER*

Sirs:

The synthesis of glutathione, one of the few naturally occurring peptides which is well characterized and readily obtainable from tissues, provides a suitable model reaction for the study of peptide bond formation. *In vivo*, glutathione is regenerated at a rapid rate.¹ We have investigated the *in vitro* synthesis of glutathione by incubating rat liver slices in the presence of glycine labeled by N¹⁵. Before incubation, small amounts of normal glutathione were added as carrier in order to facilitate the subsequent recovery of liver glutathione. The peptide was isolated in the form of its cuprous mercaptide.¹ Although the total quantity of glutathione

Aerobic Incubation of Rat Liver Slices with Labeled Glycine and Acetylglycine (32.0 Atom Per Cent Excess N¹⁵)

10 ml. of Krebs' phosphate buffer, 0.12 mm of labeled glycine or acetylglycine and 10 mg. of glutathione per gm. of wet liver.

	Atom per cent excess N ¹⁵ in glutathione after addition of	
	Glycine	Acetylglycine
hr.		
0.5	0.18	0.11
0.5		0.12
1	0.14*	0.24*
1	0.27	0.26
1	0.29	0.33

* The medium contained in addition 0.05 mm of cysteine and glutamic acid.

decreases under these conditions, significant amounts of heavy nitrogen were incorporated into glutathione, demonstrating the entrance of the added glycine into peptide linkage. It is estimated from these data that approximately 0.1 to 0.2 mg. of glutathione was synthesized per gm. of liver per hour. This rate is of the same order of magnitude as that determined by Waelsch and Rittenberg¹ for glutathione turnover in the intact rat.

With labeled acetylglycine as the test substance the quantities of N¹⁵ incorporated into glutathione were similar to those obtained with free glycine. The uptake of isotopic nitrogen by glutathione from glycine or

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¹ Waelsch, H., and Rittenberg, D., *J. Biol. Chem.*, **139**, 761 (1941)

acetylglycine was considerably smaller under anaerobic conditions. Since acetylation of amino acids as well as deacetylation occurs readily in rat liver slices,² it is not clear from the present results whether acetylaminic acids are intermediates in peptide synthesis as has been suggested.³

Small but significant amounts of N¹⁵ (0.02 to 0.03 per cent excess) were found in the material precipitable by trichloroacetic acid. This result, in confirmation of recent findings with radioactive methionine⁴ and with C¹⁴O₂,⁵ suggest that proteins can be regenerated by liver tissue *in vitro*.

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INDEX TO SUBJECTS

A

- Acetic acid:** Indole-, nicotinic acid and, plant growth inhibition, relation, *Galston*, 465
Oxal-, biosynthesis, biotin rôle, *Shive and Rogers*, 453
Acetoacetate: Isotopic, fatty acid oxidation mechanism, use in study, *Buchanan, Sakami, and Gurin*, 411
—, oxidation, biological, products, *Buchanan, Sakami, Gurin, and Wilson*, 403
Acetyl amino acid(s): Metabolism, *Bloch and Rittenberg*, 467
Acetyldehydroalanine: Hydrolysis, enzymatic, *Gonçalves, Price, and Greenstein*, 225
Acetyldehydroamino acid(s): *Escherichia coli* effect, *Fruton, Simmonds, and Smith*, 267
Aconite: Alkaloids, *Heubner and Jacobs*, 211
Adenosinetriphosphatase: Muscle, *Polis and Meyerhof*, 389
Myosin, effect, *Polis and Meyerhof*, 389
Adrenal(s): Cortex extract and testosterone propionate, liver and kidney arginase, effect, *Kochakian and Vail*, 1
— hormones, liver and kidney arginase, effect, *Kochakian and Vail*, 1
Adrenalectomy: Kidney arginase, effect, *Kochakian and Vail*, 1
Liver arginase, effect, *Kochakian and Vail*, 1
Adrenocorticotrophic hormone: Pituitary, anterior, administration effect, *Mason, Power, Ryneerson, Ciaramelli, Li, and Evans*, 223
Alanine: α -, metabolism, *Schofield and Lewis*, 373
Acetyldehydro-, hydrolysis, enzymatic, *Gonçalves, Price, and Greenstein*, 225
 β -, metabolism, *Schofield and Lewis*, 373
Phenyl-. See Phenylalanine
Albumin: Blood plasma, determination, Howe and electrophoretic methods, comparison, *Petermann, Young, and Hogness*, 379
Alkaloid(s): Aconite, *Heubner and Jacobs*, 211
Amino acid(s): Acetyl-, metabolism, *Bloch and Rittenberg*, 467
Acetyldehydro-, *Escherichia coli* effect, *Fruton, Simmonds, and Smith*, 267
Clostridium botulinum toxin, type A, crystalline, *Buehler, Schantz, and Lamanna*, 295
Hypophyseal growth hormone, *Franklin, Li, and Dunn*, 515
Liver glycogen, administration effect, *Schofield and Lewis*, 373
Nitrogen, blood plasma, determination, Folin photometric and ninhydrin manometric, comparison, *Chinard and Van Slyke*, 571
Nomenclature, *Vickery*, 237
Torulopsis utilis, utilization, *Ehrensvärd, Sperber, Saluste, Reio, and Sjernholm*, 759
Aminohippuric acid: p -, formation, liver homogenates, *Cohen and McGilvery*, 119
Anoxia: Cytochrome *c* effect, *Michel and Scheinberg*, 277
Antimony trichloride: Carotenoids, reaction, *Johnson and Baumann*, 83
Apparatus: Oxygen consumption determination, *Morrison*, 667
Arginase: Kidney, adrenalectomy, adrenal cortical hormones, testosterone propionate plus adrenal cortex extract, effect, *Kochakian and Vail*, 1
Liver, adrenalectomy, adrenal cortical hormones, testosterone propionate plus adrenal cortex extract, effect, *Kochakian and Vail*, 1

B

Bacillus: See also *Lactobacillus*

- Bacteria:** Bicarbonate utilization, biotin rôle, *Lardy, Potter, and Elvehjem*, 451
 Growth, pterins, synthetic, effect, *Daniel, Norris, Scott, and Heuser*, 689
See also Clostridium, Escherichia, Streptococcus
- Barley:** Sugar, carbon, labeled, *Aronoff, Barker, and Calvin*, 459
- Bicarbonate:** Utilization, bacteria, biotin rôle, *Lardy, Potter, and Elvehjem*, 451
- Biotin:** Bicarbonate utilization, bacteria, rôle, *Lardy, Potter, and Elvehjem*, 451
 Determination, Raney's nickel, use, *Hofmann, Winnick, and Axelrod*, 191
 Imidazolidone aliphatic acids, effect, *Dittmer and du Vigneaud*, 63
 α -Ketoglutaric acid biosynthesis, rôle, *Shive and Rogers*, 453
 Oxalacetic acid biosynthesis, rôle, *Shive and Rogers*, 453
 Oxy-, determination, Raney's nickel, use, *Hofmann, Winnick, and Axelrod*, 191
 —, yeast metabolism, *Axelrod, Flinn, and Hofmann*, 195
 Synthesis, 2-oxo-4-imidazolidinecaproic acid, effect, *Rogers and Shive*, 57
 Vaccenic acid fraction, activity, *Axelrod, Hofmann, and Daubert*, 761
- Blood:** Carbon dioxide determination, gasometric, micro-, *Scholander, Flemister, and Irving*, 173
 Gas, determination, micro-, *Scholander and Irving*, 561
 Gases, determination, gasometric, micro-, *Scholander, Flemister, and Irving*, 173
 Glycolysis, *Bird*, 493
 Lipides, phospholipide-neutral fat relationship, fasting, *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707
 Oxygen determination, gasometric, micro-, *Scholander, Flemister, and Irving*, 173
 Phospholipides, determination, *Hack*, 137
- Blood plasma:** Albumin, determination, Howe and electrophoretic methods, comparison, *Petermann, Young, and Hogness*, 379
 Amino acid nitrogen, determination, Folin photometric and ninhydrin manometric, comparison, *Chinard and Van Slyke*, 571
 Electrophoresis, injury effect, *Gjessing and Chanutin*, 657
 Factor, prothrombin activation, effect, *Ware, Guest, and Seegers*, 231
 Phosphatase, alkaline, hypophyseal growth hormone, effect, *Li, Kalman, and Evans*, 625
- Blood serum:** Magnesium, determination, molybdivanadate use, *Simonsen, Westover, and Wertman*, 39
 Proteins, differentiation, sodium sulfate solubility curves, *Majoor*, 583
 —, —, — — solubility curves and electrophoresis, comparison, *Majoor*, 583
 —, fractionation, sodium sulfate precipitation and electrophoresis, comparison, *Milne*, 595
- Brain:** Sphingolipide preparation, *Carter, Haines, Ledyard, and Norris*, 77
- Broth:** Streptomycin determination, colorimetric, *Boxer, Jelinek, and Leghorn*, 153

C

- Calcified tissue:** Phosphate, radioactive, adsorption, phosphate exchange mechanism, *Falkenheim, Neuman, and Hodge*, 713
- Calcium:** Succinic oxidase system inhibition by *d*- α -tocopheryl phosphate, effect, *Ames*, 503
- Cancer:** Tissue, β -glucuronidase activity, *Fishman and Anlyan*, 449
- Caproic acid:** 2-Oxo-4-imidazolidine-, biotin synthesis, effect, *Rogers and Shive*, 57
- Carbon:** Labeled, barley sugar, *Aronoff, Barker, and Calvin*, 459
 Radioactive, methionine methyl group-containing, synthesis, *Melville, Rachele, and Keller*, 419

- Carbon dioxide:** Blood, determination, gasometric, micro-, *Scholander, Flemister, and Irving*, 173
 Fixation, plant oxalacetate carboxylase, effect, *Gollub and Vennesland*, 233
- Carboxylase:** De-. *See* Decarboxylase
 Oxalacetate, plant, carbon dioxide fixation, effect, *Gollub and Vennesland*, 233
- Carotenoid(s):** Antimony trichloride reaction, *Johnson and Baumann*, 83
- Catheptic enzyme:** Kinetics, decarboxylase as indicator, *Frantz and Stephenson*, 359
- Cerebrospinal cord:** Sphingolipide preparation, *Carter, Haines, Ledyard, and Norris*, 77
- Citric acid:** Determination, colorimetric, micro-, *Taussky and Shorr*, 103
- Clinical preparation(s):** Streptomycin determination, colorimetric, *Boxer, Jelinek, and Leghorn*, 153
- Clostridium botulinum:** Toxin, type A, crystalline, amino acids, *Buehler, Schantz, and Lamanna*, 295
- Collagen:** Formaldehyde reaction, *Gustavson*, 531
- Cyanide:** Poisoning, cytochrome *c* effect, *Michel and Scheinberg*, 277
- Cystine:** Eggs, *Csonka, Denton, and Ringel*, 259
- Cytochrome: c**, anoxia, effect, *Michel and Scheinberg*, 277
 —, cyanide poisoning, effect, *Michel and Scheinberg*, 277

D

- Decarboxylase:** Catheptic enzyme kinetics, use as indicator, *Frantz and Stephenson*, 359
 Tyrosine, desoxyypyridoxine, phosphorylated, effect, *Beiler and Martin*, 345
- Dehydropeptidase:** Tissue, *Yudkin and Fruton*, 521
- Desoxyypyridoxine:** Phosphorylated, tyrosine decarboxylase, effect, *Beiler and Martin*, 345
- Desoxyribonucleic acid:** Determination, colorimetric, *Stumpf*, 367
- Diiodotyrosine:** Thyroxine, thyroid gland, metabolic interrelations, radioactive iodine in study, *Taurog and Chaikoff*, 49
- Diphtheria bacillus:** Porphyrin from, *Gray and Holt*, 235

E

- Egg(s):** Cystine, *Csonka, Denton, and Ringel*, 259
 Methionine, *Csonka, Denton, and Ringel*, 259
- Enzyme(s):** Acetyldehydroalanine hydrolysis, *Gonçalves, Price, and Greenstein*, 225
 Catheptic, kinetics, decarboxylase as indicator, *Frantz and Stephenson*, 359
 Peptide hydrolysis, kinetics, determination, manometric, *Zamecnik and Stephenson*, 349
 Phospholipide-splitting, nitrogenous base and phosphoric acid ester linkage, *Hanahan and Chaikoff*, 699
 Proteolytic, salmine sulfate, effect, *Portis and Altman*, 203
 , tissue, *Yudkin and Fruton*, 521
 Respiratory, tissue, determination, *Potter*, 17
 Triphosphopyridine nucleotide synthesis, *Altman and Evans*, 463
See also Adenosinetriphosphatase, Arginase, Carboxylase, Decarboxylase, Dehydropeptidase, β -Glucuronidase, Oxidase, Penicillinase, Peptidase, Phosphatase, Protaminase, Ribonucleinase, Tyrosinase
- Escherichia coli:** Acetyldehydroamino acids, effect, *Fruton, Simmonds, and Smith*, 267
 Phenylalanine utilization, *Simmonds, Tatum, and Fruton*, 91
 Tyrosine utilization, *Simmonds, Tatum, and Fruton*, 91
- Estriol:** Synthesis, partial, *Huffman*, 167
- Estrogenic substance(s):** β -Glucuronidase, relation, *Fishman*, 7
- Etiocholenol-3(α)-one-17:** Δ^5 -, urine, isolation, *Dobriner, Lieberman, Hariton, Sarett, and Rhoads*, 221

F

- Fasting:** Blood lipides, phospholipide-neutral fat relationship, *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707
 Lipides, *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707
- Fat(s):** Neutral, phospholipide-, relationship, blood lipides, fasting, *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707
 Vaccenic acid, *Geyer, Nath, Barki, Elvehjem, and Hart*, 227
- Fatty acid(s):** Oxidation, mechanism, isotopic acetoacetate in study, *Buchanan, Sakami, and Gurin*, 411
- Fetus:** Metabolism, *Goldwater and Stetten*, 723
- Food(s):** Lysine determination, microbiological, *Horn, Jones, and Blum*, 71
 Threonine determination, *Horn, Jones, and Blum*, 739
- Formaldehyde:** Collagen, reaction, *Gustavson*, 531

G

- Gas(es):** Blood, determination, gasometric, micro-, *Scholander, Flemister, and Irving*, 173
 —, —, micro-, *Scholander and Irving*, 561
 Determination, micro-, *Scholander and Evans*, 551
- Glucuronidase:** β -, cancer tissue, activity, *Fishman and Anlyan*, 449
 —, estrogenic hormone action, relation, *Fishman*, 7
- Glutamic acid:** Pteroyl-, antagonist, synthetic, *Franklin, Stokstad, Belt, and Jukes*, 427
- Glutaric acid:** α -Keto-, biosynthesis, biotin rôle, *Shive and Rogers*, 453
- Glutathione:** Synthesis, liver, *Bloch and Anker*, 765
- Glycine:** *Torulopsis utilis*, utilization, *Ehrensward, Sperber, Saluste, Reio, and Stjernholm*, 759

Glycogen: Liver, amino acid administration effect, *Schofield and Lewis*, 373

Glycolysis: Blood, *Bird*, 493

Growth: Bacteria, pterins, synthetic, effect, *Daniel, Norris, Scott, and Heuser*,

Biochemical transformations, *Rogers and Shive*, 57

Factor, liver, fractionation, *Jaffé and Elvehjem*, 287

Hormone, hypophysis, amino acids, *Franklin, Li, and Dunn*, 515

—, —, blood plasma phosphatase, alkaline, effect, *Li, Kalman, and Evans*, 625

Lactobacillus arabinosus, pterins, synthetic, effect, *Daniel, Norris, Scott, and Heuser*,

— *casei*, pterins, synthetic, effect, *Daniel, Norris, Scott, and Heuser*,

— *lactis*, liver extracts, *Shorb*, 455

—, oleic acid effect, *Hutchings and Boggiano*, 229

Plant, inhibition, indoleacetic and nicotinic acids, relation, *Galston*, 465

Streptococcus faecalis, pterins, synthetic, effect, *Daniel, Norris, Scott, and Heuser*, 689

H

Hemoglobin: Met-. See Methemoglobin

Hippuric acid: *p*-Amino-, formation, liver homogenates, *Cohen and McGilvery*, 119

Hypophysis: Growth hormone, amino acids, *Franklin, Li, and Dunn*, 515

—, —, blood plasma phosphatase, alkaline, effect, *Li, Kalman, and Evans*, 625

I

Imidazolidone: Aliphatic acids, anti-biotin activity, *Dittmer and du Vigneaud*, 63

Indoleacetic acid: Nicotinic acid and, plant growth inhibition, relation, *Galston*, 465

- Influenza:** Virus, sedimentation, viscosity, and electrophoretic studies, *Miller*, 745
- Injury:** Blood plasma electrophoresis, effect, *Gjessing and Chanulin*, 657
- Insulin:** Solutions, inactivation, *Lens*, 313
- Iodine:** Radioactive, thyroxine-diiodo-tyrosine metabolic interrelations, use in study, *Taurog and Chaikoff*, 49
- Isoserine:** Metabolism, *Schofield and Lewis*, 373

K

- Ketoglutaric acid:** α -, biosynthesis, bio-tin rôle, *Shive and Rogers*, 453
- Kidney:** Arginase, adrenalectomy, adrenal cortical hormones, testosterone propionate plus adrenal cortex extract, effect, *Kochakian and Vail*, 1

L

- Lactobacillus:** Growth, oleic acid effect, *Hutchings and Boggiano*, 229
- Lactobacillus arabinosus:** Growth, pterins, synthetic, effect, *Daniel, Norris, Scott, and Heuser*, 689
- Lactobacillus casei:** Growth, pterins, synthetic, effect, *Daniel, Norris, Scott, and Heuser*, 689
- Lactobacillus lactis:** Growth, liver extracts, *Shorb*, 455
- Lipide(s):** Blood, phospholipide-neutral fat relationship, fasting, *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707
- Fasting, *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707
- Phospho-. See Phospholipide
- Sphingo-. See Sphingolipide
- Lipositol:** Streptomycin effect, *Rhymer, Wallace, Byers, and Carter*, 457
- Liver:** *p*-Aminohippuric acid formation, *Cohen and McGilvery*, 119
- Arginase, adrenalectomy, adrenal cortical hormones, testosterone propionate plus adrenal cortex extract, effect, *Kochakian and Vail*, 1

Liver—continued:

- Extract, *Lactobacillus lactis* growth, *Shorb*, 455
- Glutathione synthesis, *Bloch and Anker*, 765
- Glycogen, amino acid administration effect, *Schofield and Lewis*, 373
- Growth factor, fractionation, *Jaffe and Elvehjem*, 287
- Urea formation, ornithine relation, *Borsook and Dubnoff*, 461
- Lysine:** Foods, determination, microbiological, *Horn, Jones, and Blum*, 71
- Proteins, determination, microbiological, *Horn, Jones, and Blum*, 71

M

- Magnesium:** Blood serum, determination, molybdivanadate use, *Simonsen, Westover, and Wertman*, 39
- Malic acid:** Phospho-, synthesis, *Friedkin and Lehninger*, 183
- Methemoglobin:** Reduction, pyridine nucleotides, rôle, *Gutmann, Jandorf, and Bodansky*, 145
- Methionine:** Eggs, *Csonka, Denton, and Ringel*, 259
- Formation, transmethylation *in vitro*, *Borsook and Dubnoff*, 247
- L-, diastereoisomeric sulfoxides from, formation, resolution, and optical properties, *Lavine*, 477
- Methyl group, oxidation *in vivo*, *MacKenzie, Chandler, Keller, Rachele, Cross, Melville, and du Vigneaud*, 757
- Synthesis, radiocarbon in methyl group, *Melville, Rachele, and Keller*, 419
- Milk:** Tocopherols, *Quaife*, 513
- , determination, chemical, *Quaife*, 513
- Whey, proteins, *Deutsch*, 437
- Muscle:** Adenosinetriphosphatase, *Polis and Meyerhof*, 389
- Myosin:** Adenosinetriphosphatase effect, *Polis and Meyerhof*, 389

N

Nicotinic acid: Indoleacetic acid and, plant growth inhibition, relation, *Galston*, 465

Nitrogen: Amino acid, blood plasma, determination, Folin photometric and ninhydrin manometric, comparison, *Chinard and Van Slyke*, 571

Nomenclature: Amino acids, *Vickery*, 237

Nucleic acid: Desoxyribo-, determination, colorimetric, *Stumpf*, 367

Ribo-, yeast, ribonucleinase, hydrolysis, *Loring, Carpenter, and Roll*, 601

—, —, ribonucleinase-resistant fractions, hydrolysis and preparation, *Loring, Carpenter, and Roll*, 601

Nucleotide(s): Pyridine, methemoglobin reduction, rôle, *Gutmann, Jandorf, and Bodansky*, 145

Triphosphopyridine, synthesis, enzymatic, *Allman and Evans*, 463

O

Oil(s): Vaccenic acid, *Geyer, Nath, Barki, Elvehjem, and Hart*, 227

Oleic acid: *Lactobacillus* growth, effect, *Hutchings and Boggiano*, 229

Oligophrenia: Phenylpyruvic, metabolic error, position, *Jervis*, 651

Ornithine: Urea formation, liver homogenate, relation, *Borsook and Dubnoff*, 461

Oxalacetate: Carboxylase, plant, carbon dioxide fixation, effect, *Gollub and Vennesland*, 233

Oxalacetic acid: Biosynthesis, biotin rôle, *Shive and Rogers*, 453

Oxidase: Succinic, system, inhibition by *d*- α -tocopheryl phosphate, calcium effect, *Ames*, 503

Oxo-4-imidazolidinecaproic acid: 2-, biotin synthesis, effect, *Rogers and Shive*, 57

Oxybiotin: Determination, Raney's nickel, use, *Hofmann, Winnick, and Axelrod*, 191

Yeast metabolism, *Axelrod, Flinn, and Hofmann*, 195

Oxygen: Blood, determination, gasometric, micro-, *Scholander, Flemister, and Irving*, 173

Consumption, determination, apparatus, *Morrison*, 667

P

Penicillinase: Isolation, *Morgan and Campbell*, 337

Production, *Morgan and Campbell*, 337

Peptidase: Dehydro-, tissue, *Yudkin and Fruton*, 521

Peptide(s): Bond, synthesis, *Cohen and McGilvery*, 119

Hydrolysis, enzymatic, kinetics, determination, manometric, *Zamecnik and Stephenson*, 349

Synthesis, *in vivo*, *Friedberg, Winnick, and Greenberg*, 763

Phenylalanine: *Escherichia coli*, utilization, *Simmonds, Tatum, and Fruton*, 91

Phenylpyruvic oligophrenia: Metabolic, error, position, *Jervis*, 651

Phosphatase: Adenosinetri-, muscle, *Polis and Meyerhof*, 389

—, myosin, effect, *Polis and Meyerhof*, 389

Alkaline, blood plasma, hypophyseal growth hormone, effect, *Li, Kalman, and Evans*, 625

Phosphate(s): Calcified tissue, radioactive phosphate adsorption, *Falkenheim, Neuman, and Hodge*, 713

Phospholipide(s): Blood, determination, *Hack*, 137

-Neutral fat relationship, blood lipides, fasting, *Hodge, MacLachlan, Bloor, Welch, Kornberg, and Falkenheim*, 707

-Splitting enzyme, nitrogenous base and phosphoric acid ester linkage, *Hanahan and Chaikoff*, 699

Phosphomalic acid: Synthesis, *Friedkin and Lehninger*, 183

Phosphorylation: Oxidative, *Potter*, 17

Pituitary: Anterior, adrenocorticotrophic hormone, administration effect, *Mason, Power, Ryneerson, Ciaramelli, Li, and Evans*, 223

See also Hypophysis

- Plant:** Growth, inhibition, indoleacetic and nicotinic acids, relation, *Galston*, 465
- Oxalacetate carboxylase, carbon dioxide fixation, effect, *Gollub and Vennesland*, 233
- Porphyrim:** *Diphtheria bacillus*, *Gray and Holt*, 235
- Potassium:** Biological materials, determination as potassium phosphotungstate, *Folch and Lauren*, 539
- Proline:** *Torulopsis utilis*, utilization, *Ehrensward, Sperber, Saluste, Reio, and Stjernholm*, 759
- Protaminase:** Salmine sulfate, effect, *Portis and Altman*, 203
- Protein(s):** Blood serum, differentiation, sodium sulfate solubility curves, *Majoer*, 583
- — — sulfate solubility curves and electrophoresis, comparison, *Majoer*, 583
- — — fractionation, sodium sulfate precipitation and electrophoresis, comparison, *Milne*, 595
- Lysine determination, microbiological, *Horn, Jones, and Blum*, 71
- Milk whey, *Deutsch*, 437
- Threonine determination, *Horn, Jones, and Blum*, 739
- Tyrosinase effect, *Sizer*, 303
- Proteolysis:** Enzymes, salmine sulfate, effect, *Portis and Altman*, 203
- , tissue, *Yudkin and Fruton*, 521
- Prothrombin:** Activation, blood plasma factor, effect, *Ware, Guest, and Seegers*, 231
- Pterin(s):** Synthetic, bacterial growth, effect, *Daniel, Norris, Scott, and Heuser*, 689
- , *Lactobacillus casei* and *Lactobacillus arabinosus* growth, effect, *Daniel, Norris, Scott, and Heuser*, 689
- , *Streptococcus faecalis* growth, effect, *Daniel, Norris, Scott, and Heuser*, 689
- Pteroylglutamic acid:** Antagonist, synthetic, *Franklin, Stokstad, Belt, and Jukes*, 427
- Pyridine:** Nucleotides, methemoglobin reduction, rôle, *Gutmann, Jandorf, and Bodansky*, 145
- Triphospho-, nucleotide, synthesis, enzymatic, *Altman and Evans*, 463
- Pyridoxamine phosphate:** Microbiological activity and occurrence, *Rabinowitz and Snell*, 643
- Pyridoxine:** Desoxy-, phosphorylated, tyrosine decarboxylase, effect, *Beiler and Martin*, 345
- ### R
- Respiratory enzyme(s):** Tissue, determination, *Potter*, 17
- Ribonucleic acid:** Yeast, ribonucleinase resistant fractions, hydrolysis and preparation, *Loring, Carpenter, and Roll*, 601
- Ribonucleinase:** -Resistant fractions, yeast ribonucleic acid, hydrolysis and preparation, *Loring, Carpenter, and Roll*, 601
- Yeast ribonucleic acid hydrolysis, *Loring, Carpenter, and Roll*, 601
- ### S
- Salmine sulfate:** Enzymes, proteolytic, effect, *Portis and Altman*, 203
- Protaminase effect, *Portis and Altman*, 203
- Serine:** Iso-, metabolism, *Schofield and Lewis*, 373
- Metabolism, *Schofield and Lewis*, 373
- Sphingolipide(s):** Biochemistry, *Carter, Haines, Ledyard, and Norris*, 77
- Brain, preparation, *Carter, Haines, Ledyard, and Norris*, 77
- Cerebrospinal cord, preparation, *Carter, Haines, Ledyard, and Norris*, 77
- Spinal cord:** See Cerebrospinal cord
- Staphisine:** *Huebner and Jacobs*, 211
- Hydrocarbon, *Huebner and Jacobs*, 211
- Steroid(s):** 16-Substituted, *Huffman*, 167
- Streptococcus faecalis:** Growth, pterins, synthetic, effect, *Daniel, Norris, Scott, and Heuser*, 689
- Vitamin B₆ determination, use in, *Rabinowitz and Snell*, 631

- Streptomycin**: Broth, determination, colorimetric, *Bozer, Jelinek, and Leghorn*, 153
 Clinical preparations, determination, colorimetric, *Bozer, Jelinek, and Leghorn*, 153
 Lipositol effect, *Rhymer, Wallace, Byers, and Carter*, 457
 Urine, determination, colorimetric, *Bozer, Jelinek, and Leghorn*, 153
Succinic oxidase: System, inhibition by *d*- α -tocopheryl phosphate, calcium effect, *Ames*, 503
Sugar: Barley, carbon, labeled, *Aronoff, Barker, and Calvin*, 459

T

- Tellurium**: *DeMeio and Henriques*, 609
 Tissue, excretion and distribution, radioactive isotope in study, *DeMeio and Henriques*, 609
Testosterone propionate: Adrenal cortex extract and, liver and kidney arginase, effect, *Kochakian and Vail*, 1
Thiouracil: Determination, *Olson, Ely, and Reineke*, 681
Threonine: Foods, determination, microbiological, *Horn, Jones, and Blum*, 739
 Proteins, determination, microbiological, *Horn, Jones, and Blum*, 739
Thyroid: Thyroxine-diiodotyrosine metabolic interrelations, radioactive iodine in study, *Taurog and Chaikoff*, 49
Thyroxine: Diiodotyrosine, thyroid gland, metabolic interrelations, radioactive iodine in study, *Taurog and Chaikoff*, 49
Tocopherol(s): Milk, *Quaife*, 513
 —, determination, chemical, *Quaife*, 513
Tocopheryl phosphate: *d*- α -, succinic oxidase system inhibition by, calcium effect, *Ames*, 503
Torulopsis utilis: Amino acid utilization, *Ehrensward, Sperber, Saluste, Reio, and Stjernholm*, 759

Torulopsis utilis—continued:

- Glycine utilization, *Ehrensward, Sperber, Saluste, Reio, and Stjernholm*, 759
 Proline utilization, *Ehrensward, Sperber, Saluste, Reio, and Stjernholm*, 759
Toxin: *Clostridium botulinum*, type A, crystalline, amino acids, *Buehler, Schantz, and Lamanna*, 295
Triphosphopyridine: Nucleotide synthesis, enzymatic, *Altman and Evans*, 463
Tyrosinase: Proteins, effect, *Sizer*, 303
Tyrosine: Decarboxylase, desoxypyridoxine, phosphorylated, effect, *Beiler and Martin*, 345
 Diiodo-, thyroxine, thyroid gland, metabolic interrelations, radioactive iodine in study, *Taurog and Chaikoff*, 49
Escherichia coli, utilization, *Simmonds, Tatum, and Fruton*, 91

U

- Uracil**: Thio-, determination, *Olson, Ely, and Reineke*, 681
Urea: Formation, liver homogenate, ornithine relation, *Borsook and Dubnoff*, 461
Urine: Δ^5 -Etiocholenol-3(α)-one-17, isolation, *Dobriner, Lieberman, Hariton, Sarett, and Rhoads*, 221
 Streptomycin determination, colorimetric, *Bozer, Jelinek, and Leghorn*, 153

V

- Vaccenic acid**: Fats, *Geyer, Nath, Barki, Elvehjem, and Hart*, 227
 Fraction, biotin activity, *Azel od, Hofmann, and Daubert*, 761
 Oils, *Geyer, Nath, Barki, Elvehjem, and Hart*, 227
Virus: Influenza, sedimentation, viscosity, and electrophoretic studies, *Miller*, 745

Vitamin (s) : A, decomposition, oxidative,
Bolomey, 323, 331
 —, oxidation and irradiation effect,
Bolomey, 323
 —, oxidized, spectrophotometry, *Bolomey*, 331
B., *Rabinowitz and Snell*, 631, 643
 —, determination, *Streptococcus faecalis* use, *Rabinowitz and Snell*, 631
 E. *See also* Tocopherol
 H. *See also* Biotin

W

Whey; Milk, proteins, *Deutsch*, 437

Y

Yeast: Oxybiotin metabolism, *Axelrod, Flinn, and Hofmann*, 195
 Ribonucleic acid, ribonucleinase hydrolysis, *Loring, Carpenter, and Roll*, 601
 — —, ribonucleinase-resistant fractions, hydrolysis and preparation, *Loring, Carpenter, and Roll*, 601
See also *Torulopsis*

